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Title of Dissertation: "The Regulation of Endogenous Glutamate and GABA Release from *In Vitro* Preparations of Rat Striatum"

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Doctor of Philosophy Degree
19 September 1997

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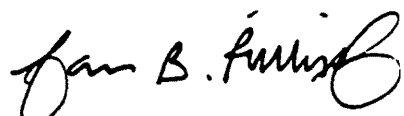

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ABSTRACT

The release of the major excitatory and inhibitory neurotransmitters, glutamate and GABA, from rat caudate-putamen (CP) tissue slices, and GABA release from fetal rat primary striatal cultures, were studied to ascertain key mechanisms underlying the regulation of their release. An improved, highly sensitive method of high performance liquid chromatography and electrochemical detection (LCED) was developed to enable the reliable measurement of these endogenous amino acids from tissue release extracts. The assay permits detection of endogenous amino acids from one milligram of CP tissue *in vitro*. Release of both amino acids from CP tissue was evoked by elevated levels of potassium, while the potassium channel blocker, 4-aminopyridine (4-AP), selectively released GABA. Glu was selectively released by the excitatory amino acid (EAA) receptor agonist, N-methyl-D-aspartate (NMDA), independently of external Ca^{2+} concentration. Ca^{2+}-dependent release of Glu and GABA by potassium- and 4-AP-evoked depolarization was shown to be solely dependent on external Ca^{2+} influx through the P-type Ca^{2+} channel ($P<0.005$); blockade of L- and N- types of Ca^{2+} channels did not reduce release of either amino acid. Potassium and 4-AP also evoked Ca^{2+}-dependent release of GABA from the striatal cultures; GABA was also released by Glu, through a Ca^{2+}-independent process. Activation of D_1 dopamine receptors elicited a significant stimulation of GABA release from striatal cultures ($P<0.01$), but no dopaminergic effects were observed on release of either amino acid in CP tissue slices. Potassium (50 mM KCl) stimulated release of GABA from the cultures was shown to be significantly increased by bicuculline ($P<0.05$), the $GABA_A$ receptor antagonist.

These studies suggest that NMDA or Glu stimulation of *in vitro* Glu and GABA release is most likely mediated through depolarization-evoked reversal of the plasma membrane amino acid transporters. Dopamine has a facilitatory influence on striatal GABA release, and autoreceptor regulation of GABA release is through the $GABA_A$

receptor. The experimental conditions under which Glu and GABA were analyzed, provide an efficient means of studying regulatory influences of striatal amino acid release.

THE REGULATION OF ENDOGENOUS GLUTAMATE AND GABA RELEASE FROM *IN VITRO* PREPARATIONS OF RAT STRIATUM

by

James B. Phillips, Jr.

Dissertation submitted to the Faculty Committee from Departments of Pharmacology and Neurology and the Neuroscience Program of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy
1997

Dedication

To my wonderful and loving wife, Rebecca Rohrer.

Acknowledgments

Thanks Rebecca for your continued support and encouragement.

I would like to acknowledge my mentor, Dr. Brian Cox. I will value his guidance and advice throughout my scientific career.

I would like to thank the additional members of my dissertation committee, Drs. Tom Cote, Cinda Helke, Joe Long and Ajay Verma. I greatly appreciate their individual attention devoted to the successful completion of my graduate degree.

Special appreciation is extended to Dr. Cote for serving as chairman of my committee. His enthusiasm was an inspiration throughout my tenure at USUHS.

Additional thanks to Dr. Helke for her keen scientific and professional advice.

I would like to especially recognize my parents - I greatly appreciate their support and inspiring me to pursue my dreams.

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Abbreviations of commonly used terms

4-AP	4-aminopyridine
5-HT	serotonin
ACh	acetylcholine
AgTx IVa	ω-agatoxin IVa
AMPA	α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
ANOVA	analysis of variance
BG	basal ganglia
CnTx GVIA	ω-conotoxin GVIA
CP	caudate-putamen
DA	dopamine
DYN	dynorphin
EAA	excitatory amino acid
ENK	enkephalin
GABA	γ-aminobutyric acid
GABA-T	GABA transaminase
GAD	glutamic acid decarboxylase
Glu	glutamate
HPLC	high performance liquid chromatography
HRP	horse radish peroxidase
L-trans-2,4-PDC	L-trans-pyrollidine-2,4-dicarboxylic acid
LCED	liquid chromatography-electrochemical detection
LPS	lateral pallidal segment
MKB	modified Krebs-HEPES buffer
MPS	medial pallidal segment
NAcc	nucleus accumbens
NMDA	N-methyl-D-aspartate
OPA-SO_3	o-phthalaldehyde-sodium sulphite
PBS	phosphate buffered saline
PSC	fetal rat primary striatal cultures
SEM	standard error of the mean
SN	substantia nigra
SNc	substantia nigra pars compacta
SNr	substantia nigra pars reticulata
SP	substance P

Abbreviations continued

STN	.....	subthalamic nucleus
VP	.....	ventral pallidum
VTA	.....	ventral tegmental area

INTRODUCTION

A. Nuclear subdivision of the basal ganglia

The basal ganglia (BG) are a mass of subcortical nuclei arranged into multiple internal loops that use various neurotransmitters to communicate between subdivisions. The major divisions of the BG are the corpus striatum and the amygdaloid nuclear complex. The corpus striatum includes the caudate nucleus, the nucleus accumbens (NAcc), the putamen, and the globus pallidus. Together, the caudate, NAcc and putamen form the neostriatum and are collectively referred to as the striatum. The BG is also divided anatomically into dorsal and ventral aspects. The dorsal aspect of the BG primarily consists of the caudate, putamen and globus pallidus; they extend ventrally below the anterior commissure to form the ventral striatum and the ventral pallidum (Heimer et al., 1991). The ventral extensions of the corpus striatum and the amygdaloid nuclear complex form the ventral tier of the BG which is associated with limbic functions of the BG circuitry. The dorsal, non-limbic portion is primarily associated with motor activity. For clarity, within the context of this text, I will generally refer to the dorsal and ventral aspects of the striatum as the caudate-putamen (CP) and NAcc, respectively, and "striatum" refers to both dorsal and ventral neurons of the caudate, putamen and NAcc, collectively.

1. *Dorsal (nonlimbic) basal ganglia*

The most studied aspect of the BG circuitry is the dorsal region through its association with planning, initiation, and learning of motor movement. Muscle movement disorders such as Huntington's Chorea and Parkinson's Disease stem from abnormal connections between subdivisions and nuclei of the BG that normally form a circuit of information relayed by various neurotransmitter interactions (Albin et al., 1989). The anatomy of the dorsal BG is often referred to as a subcortical motor loop or extrapyramidal system which possesses a basic circuit superimposed within the output of the motor cortex (Graybiel, 1990; Cedarbaum and Schleifer, 1990; and Riederer et al., 1992).

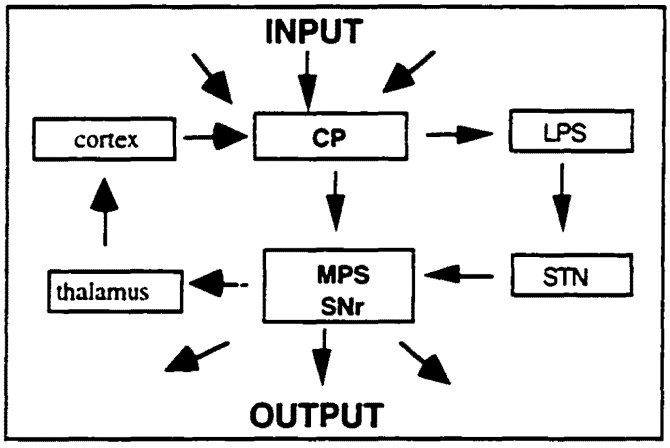


FIGURE 1 - The basal ganglia loop showing both direct and indirect pathways (Adapted from Gross Anatomy III notes, Dept of Anatomy, USUHS).

In figure 1, this loop is shown along with the key nuclei involved in the normal flow of neuronal information. The loop receives a majority of its signals from the cortex, shuffles them through an inter-connected network of neurons and nuclei, then sends these cortical signals back to specific areas of the cortex in a defined pattern to regulate motor memory and motor planning (Graybiel, 1990; and Graybiel et al., 1993).

The CP (and NAcc) is the primary receptive structure of the BG which receives the majority of its information from the substantia nigra pars compacta (SNc) and all major areas of the cortex (Kemp et al., 1970). The CP sends efferent projections to the primary output neurons of the BG, the medial pallidal segment (MPS) and the substantia nigra pars reticulata (SNr), through both direct and indirect pathways. The direct pathway sends inhibitory striatonigral and striatoendopeduncular projections, while the indirect pathway begins by sending inhibitory projections to the lateral pallidal segment (LPS). The LPS then projects inhibitory input to the subthalamic nucleus (STN), and the STN in turn sends stimulatory projections to the output nuclei of the BG. The two pathways allow for both an inhibitory input into the MPS and SNr through the direct route and a stimulatory input through the indirect pathway. Output from the dorsal BG, via the MPS and SNr, leads to the ventrolateral thalamic nucleus and then the primary motor cortex before the descending motor pathway is activated.

2. Ventral (limbic) basal ganglia

The three major areas of the ventral BG are the amygdala, the NAcc, and the ventral pallidum (VP). Together these structures, though function as an integral part of the limbic

system, do not form a distinct subcortical circuit loop like the dorsal BG as seen in Figure 1. The amygdala differs from other BG nuclei in that it sends efferents to numerous nuclei located outside the BG, while the MPS and SNr, the major output nuclei of the BG, send most of their fibers to the thalamus and superior colliculus. The amygdala efferents projecting beyond the BG extend to the thalamus, the hypothalamus, the septal area, the hippocampus, subiculum, substantia innominata, entorhinal cortex, the somatosensory cortex and constitute the stria terminalis. The amydaloid nuclear complex also sends descending fibers to the parabrachial nuclei, the solitary fasciculus, and dorsal motor nucleus of the vagus (Carpenter, 1991). Within the BG, the amygdala projects to the ventral striatum, including the NAcc and the olfactory tubercle.

The principle efferent projections of the NAcc are GABAergic and extend primarily to the VP and mesencephalic regions including the ventral tegmental area (VTA). These projections are involved in two distinct reciprocal circuits. Accumbal projections to the VP and the VTA are reciprocated by pallidal GABAergic efferents and tegmental GABAergic and dopaminergic projections (Van Bockstaele and Pickel, 1995). These circuits serve as feedback loops whereby transmitter release within the ventral tier of the BG is under local control. Afferents of the NAcc arise primarily from the cortex, VP, amygdala, and VTA. The temporal, prefrontal, and parietal association cortices, as well as the amygdala projections are stimulatory. The VP is thought to project GABA, and the VTA sends stimulatory and inhibitory projections through GABA and dopamine (DA) neurons; the dopaminergic pathway is implicated in the reinforcing properties of abused drugs (Fuxe et al., 1985; Groenewegen et al., 1994; and Self and Nestler, 1995).

B. Major neurons and neurotransmitters of the basal ganglia

When considering the role that the BG play in the control over motor movement and relaying behavioral information, it seems likely that this subcortical mass of nuclei would have a remarkable number of neurotransmitters and receptors. Indeed, the BG circuitry is

rich in classical neurotransmitters such as DA, GABA, glutamate (Glu), acetylcholine (ACh) and serotonin (5-HT). Some of these transmitters are co-located with peptide neurotransmitters such as the opiates, substance P, somatostatin, cholecystokinin, vasoactive intestinal polypeptide, and neurotensin (Guttman, 1987; and Semba et al., 1987). The transmitters arise from nerve terminals, interneurons and dendrites that are located throughout the BG. Because the striatum receives most of the BG input coming primarily from the cortex, thalamus, substantia nigra, and amygdala, it contains the most diverse amount of transmitters and receptors; while, the striatal efferent targets, the globus pallidus and substantia nigra, are less heavily innervated by different transmitters. The amygdala, being an integral part of the limbic system, also contains a great diversity of transmitters and receptors. Peptide transmitters are especially high, and the amount of somatostatin in the amygdala is among the highest in the brain (Carpenter, 1991). The transmitters discussed below will primarily focus on their interactions within the striatum, because the project centers on Glu and GABA release in this area, although the circuitry of the BG cannot be ignored.

1. GABA

The predominant neurotransmitter within the BG is GABA (Graybiel, 1990). It is estimated that over eighty percent of neurons in the NAcc and over ninety percent in the dorsal striatum use GABA as their neurotransmitter (Smith and Sharp, 1994b; and Maneuf et al., 1994), and most neurons of the globus pallidus and SNr are GABAergic (Graybiel, 1990). Most GABAergic neurons are medium-size, spiny type, while a few, specifically within the striatum, are medium-sized, aspiny interneurons (Fonnum et al., 1977). Retrograde transport of HRP with Golgi impregnation under electron microscopy has established that almost all projection neurons of the striatum are the medium-size spiny

type with dendrites densely covered with spines as shown in figure 2 (Smith and Bolam, 1990). In the striatum, GABA is located in two distinct classes of neurons; one containing GABA, substance P (SP), and dynorphin (DYN) which projects to the SNr and the medial pallidal segment and the other which contains GABA co-localized with enkephalin (ENK) and projects to the lateral pallidal segment (Maneuf et al., 1994) (see figure 2). These two types of GABAergic neurons are laterally connected through recurrent collaterals arising from their striatal efferent medium spiny axons as indicated in figure 2 (Smith and Bolam, 1990). The GABA/SP/DYN cell bodies also project interneurons which act on cholinergic neurons within the CP (Di Chiara et al., 1994).

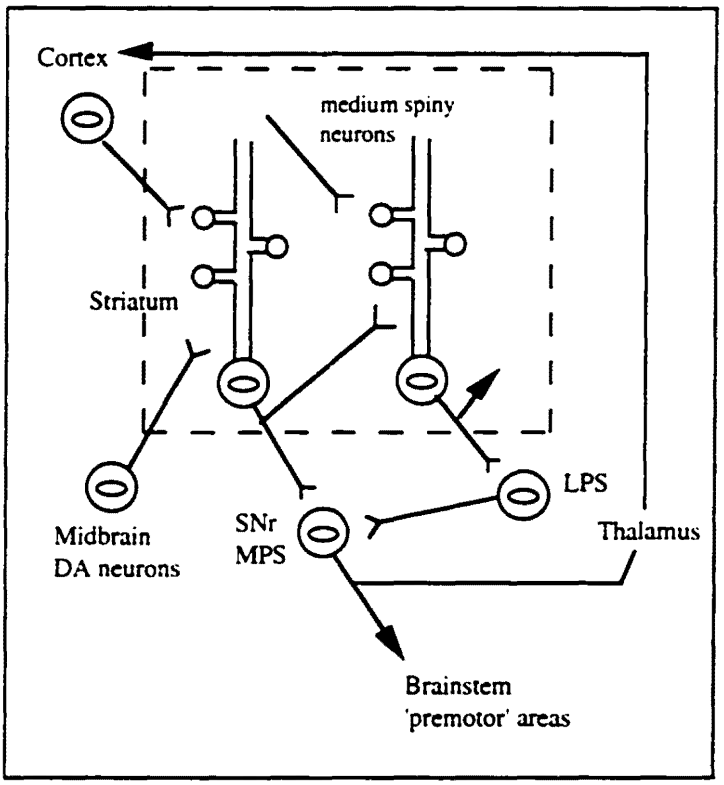


FIGURE 2 - Medium spiny GABAergic neurons of the striatum and the basal ganglia loop. Adapted from Smith and Bolam, 1990.

GABA is thought to be released by several means in the BG. In the GP and SN, GABA is released from the terminals of striatal axons, and is under presynaptic control by mainly autoinhibition. GABA release in the striatum is from terminals of recurrent collaterals, interneurons and proximal dendrites. Dendritic release is thought to exist because GABA release from striatal slices is tetrodotoxin resistant (Glowinski et al., 1994). Glowinski et al (1994) have shown that NMDA receptor activation can stimulate GABA release from collaterals and dendrites in the striatum, and Smith and Bolam (1990) have

shown that the dendrites of medium-size spiny GABA neurons are directly innervated with DA, Glu, ACh, and SP terminals.

2. Glutamate

Glutamate innervates the striatum through both corticostriatal and thalamostriatal fibers and innervates the SNr and GP through subthalamic efferent fibers. Anterograde tracing and immunocytochemistry have shown that Glu input into the striatum is fairly disperse throughout the caudate nucleus, putamen, and NAcc, but that synaptic contact is selective for specific neurons in the striatum. Glutamatergic fibers from the parafascicular nucleus of the thalamus make direct contact with intrinsic cholinergic neurons in both the dorsal and ventral striatum, while corticostriatal neurons presumably make contact with GABAergic output neurons of the striatum and DA neurons dispersed throughout the striatum (Meredith and Wouterlood, 1990; and Lapper and Bolam, 1992). Glu fibers from the STN which innervate the output nuclei of the BG is part of the indirect pathway of the BG circuitry. The STN also acts back on the LPS and is thought to function as a feedback mechanism (Smith et al., 1994). Smith and Parent (1988) have shown that the majority of subthalamic efferent fibers are glutamatergic that act on the SNr and GP and are themselves innervated by GABAergic afferents of the LPS.

Through AMPA and NMDA receptors Glu mediates excitatory activity on most neurons of the striatum. The activated AMPA receptor mediates a fast stimulatory response, and the NMDA response is considered slower and referred to as 'modulatory' (Di Chiara et al., 1994). The NMDA receptor is a voltage-gated ion channel that is under a Mg^{2+} blockade; it requires a depolarization of the neuron to overcome the block. Therefore, Glu neurons innervating the striatum require AMPA receptors or excitatory cholinergic and dopaminergic input to depolarize the neuron and relieve the Mg^{2+} block (Di Chiara et al., 1994). DA afferents converge with Glu terminals on dendritic spines of GABA neurons to yield a combined influence on GABA release (Cepeda et al., 1993; and Kötter, 1994), although they do not necessarily produce a synergistic effect. Cepeda et al

(1993) showed that D_1 DA receptor stimulation enhances NMDA receptor responses, while D_2 DA receptors inhibit both NMDA and AMPA receptor increases in electrical potential in rat neostriatal slices.

3. Dopamine

Dopamine innervates the striatum from the retrorubral nucleus, the SNc, and the VTA (cell groups A8, A9 and A10, respectively) through the medial forebrain bundle. Although DA from all three groups acts on the entire dorsal and ventral striatum (Groenewegen et al., 1994), the SNc projections are more closely associated with the dorsal striatum, and the VTA projections are associated with the more ventral, limbic striatum (Fuxe et al., 1985; and Graybiel, 1990). The amygdala is also innervated by all three DA cell groups. The GP is not heavily innervated by DA neurons, so DA has little direct effect on pallidal activity, although it has significant indirect effects. DA terminals and receptors are heavily dispersed throughout the CP and NAcc, and through selective receptors and synapse localization, DA can effect most neurotransmitters located in these nuclei. Approximately 21% of the total axon terminals in the striatum are dopaminergic (Kötter, 1994), and these terminals innervate the GABA medium spiny neurons at more distal areas of the dendrites, areas where Glu terminals synapse with the GABA neurons. The innervation is symmetric on the striatal output neurons, meaning that only one side of the dendritic shaft is in contact with the DA neurons (Smith and Bolam, 1990). Of the five types of DA receptors, D_1 and D_2 receptors are thought to predominate in the striatum. D_1 DA receptors are found primarily on GABA/SP/DYN neurons of the direct BG pathway and on the intrinsic cholinergic neurons. D_2 DA receptors are located primarily on the GABA neurons containing ENK that project to the LPS and on most of the presynaptic terminals of DA, ACh, and Glu neurons (Di Chiara et al., 1994) as indicated in figure 3 on the following page.

The D_1 receptor is stimulatory, and therefore stimulates GABA release to the SNr and MPS, while the D_2 receptor inhibits the release of GABA to the LPS. This, along with excitatory Glu input, is important in maintaining the direct circuit pathway of the BG which serves to inhibit firing of GABA neurons of the output nuclei, and therefore increases the firing of efferents from the motor thalamus (Graybiel, 1990).

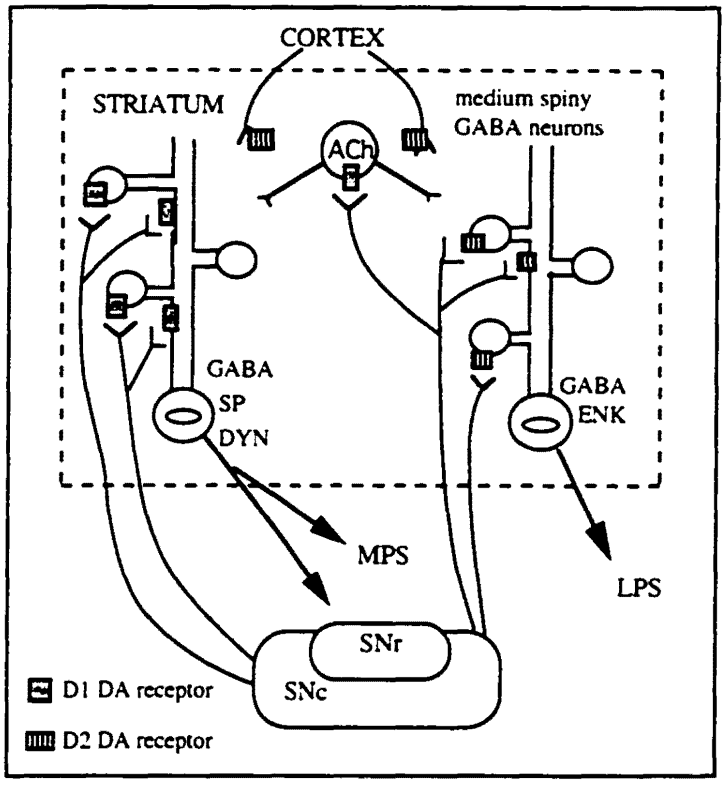


FIGURE 3 - Dopamine (DA) innervation and specific receptor localization within the striatum. Spiny neurons of the direct pathway possess mostly D1 DA receptors, as do the cholinergic interneurons, while spiny neurons of the indirect pathway possess D2 DA receptors. Not pictured is that most DA terminals contain D2 DA autoreceptors and NMDA/AMPA receptors.

4. Acetylcholine

Within the BG, ACh is found in the CP and NAcc where its large aspiny interneurons make up approximately 1-2 percent of the total neuronal population of the entire striatum (Graybiel et al., 1986; and Stoof et al. 1992). Its major intrinsic target is the GABAergic output nuclei (Izzo and Bolam, 1988), and it receives direct input from ascending DA neurons, cortical and thalamic Glu neurons, and intrinsic SP projections (Kubota et al., 1987; Chang, 1988; Lapper and Bolam, 1992; Bolam et al, 1986; and Gerfen, 1991). Opiate peptides are also thought to tonically control ACh activity in the striatum (Sandor et al., 1992), and the intrinsic ACh may control its own release through M1, M2, and M4 muscarinic receptors located on the large aspiny interneurons (Bernard et al., 1992).

ACh, like DA, is both stimulatory and inhibitory toward GABA release from the CP. Both striatopallidal and striatonigral GABAergic neurons express M1 and M4 muscarinic receptors (Bernard et al., 1992), but through muscarinic control of c-fos expression in selective striatal neurons, M1 and M4 receptors are thought to differentially influence the two types of medium-sized spiny GABAergic neurons (Di Chiara et al., 1994). Bernard et al (1993) have shown that the muscarinic receptor agonist oxotremorine preferentially stimulates c-fos expression in ENKergic striatopallidal efferent neurons, presumably through M1 receptor stimulation which stimulates phosphoinositol turnover. They relate this to muscarinic tone in the striatum, meaning that although 60% of the ENK/GABA neurons express both m1 and m4 receptor genes, the other 40% express only the m1 gene. They also showed that most of the atropine (a muscarinic receptor antagonist) stimulated c-fos expression occurred in GABA/SP striatonigral neurons, presumably through blocking M4 inhibitory receptors. Di Chiara et al (1994) therefore propose that M1 receptor activity predominates on ENK neurons while M4 receptors are primarily responsible for the cholinergic activity on the GABA/SP/DYN neurons. Therefore, ACh and DA mediate effects opposite one another to influence the release of GABA from the CP through $M1/D_2$ receptor activity on GABA/ENK striatopallidal neurons and $M4/D_1$ receptor activity on GABA/SP/DYN striatonigral neurons.

5. Opiate peptides

The opiate peptides ENK and DYN are separately found co-located with GABA in the medium-size spiny neurons of the striatum. As previously indicated in figure 3 on page 8, the GABA/DYN neurons, also co-located with SP, make up the output neurons of the BG direct pathway sending projections to the SNr and the MPS. The GABA/ENK neurons project to the LPS and initiate the indirect pathway of the BG motor loop (Graybiel, 1990). It is likely that endogenous DYN and ENK release in the striatum arise from the recurrent collaterals extending from the striatal spiny projection neurons. The opiate peptides are thought to also be located in the GP and SNr, but only in the terminals of striatal efferent

fibers (Abou-Khalil et al., 1984). Of the three major classes of opioid receptors, μ, δ and κ, all are found, to various degrees, dispersed throughout all BG nuclei. In the rat, μ-receptors are found heaviest in the amygdala, δ are very densely distributed in the striatum, and the κ-receptors are heaviest in the NAcc and part of the amygdala (Mansour et al., 1995). Having both opiate peptides and receptors located in the BG, indicate that ENK and DYN may function in the routine control of neurotransmission through this mass of subcortical neurons.

Opiate peptides are primarily inhibitory toward neurons on which they act, inhibiting both the neuron firing rate and the amount of transmitter released by the neuron (North, 1986). ENK primarily acts at the μ- and δ- type of opiate receptor, while DYN is thought to be the primary endogenous agonist of the κ-receptor. Opiate receptor agonists inhibit neurotransmitter release from neurons throughout the BG. In the mammalian striatum, exogenously applied opiates inhibit the release of ACh, DA, Glu and GABA through an opiate receptor mediate action (Mulder et al., 1984; Werling et al., 1988; and Jiang and North, 1992). GABA release is also inhibited in the GP by opioids (Dewar et al., 1987; and Maneuf et al., 1994), presumably because opiate receptors are located on the striatopallidal presynaptic GABAergic spiny neurons (Abou-Khalil et al., 1984).

Opiate receptor agonists inhibited the release of [^{3}H]-ACh by 50 to 60% of control from rat striatal tissue through the δ-type receptor (Mulder et al., 1984; and Schoffelmeer et al., 1992, respectively), and the release of endogenous ACh primarily through the μ-type receptor and not the δ-type (Lapchak et al., 1989). It has not been suggested, though, if the opiate receptors are located on either the presynaptic terminals of the aspiny cholinergic interneurons or their cell bodies. Although, in the NAcc, μ- and δ- receptors are presumably located on cholinergic terminals (Heijna et al., 1992). The κ-receptor is thought to be located presynaptically on nigrostriatal DA neurons and exert an inhibitory influence on DA release in the striatum (Werling et al., 1988), but selective κ-receptor agonists have no significant effect on DA release in the GP (Dewar et al., 1987). Heijna et

al (1992) have shown also that the κ-receptor agonist U50488 inhibits [^{3}H]-DA release from the rat NAcc, whereas μ- and δ-receptor agonist inhibit [^{14}C]-ACh release but had no effect on DA release.

Both μ- and δ- type of opioid receptors are thought be located on Glu terminals in the striatum arising from cortical excitatory input (Jiang and North, 1992). μ- and δ-, but not κ-, receptor stimulation inhibits synaptic potentials in the striatum mediated by excitatory amino acids; this is interpreted to mean that the opioids are presynaptically inhibiting Glu release (Jiang and North, 1992). It has also been suggested that subtypes of opiate receptors are differentially expressed on the two types of striatal GABAergic neurons (Noble and Cox, 1995). The D_1 DA receptor is preferentially expressed on the striatonigral/endopeduncular neurons, and the A_{2a} adenosine receptor is found primarily on the striatopallidal neurons; both receptors stimulate adenylyl cyclase activity. Noble and Cox (1995) have shown that in rat striatal tissue, μ- and δ_2- receptors specifically inhibit cyclase activity stimulated by D_1 DA receptors, and δ_1- and δ_2 -receptors inhibit cyclase activity stimulated by A_{2a} adenosine receptors. This has allowed for the proposed opioid receptor localization on the striatal medium spiny neurons as depicted in Figure 4. μ- and δ_2 -receptors are thought to be located with D_1 DA receptors on striatonigral/endopeduncular neurons, while the δ_1- and δ_2 -subtypes are co-expressed on striatopallidal neurons.

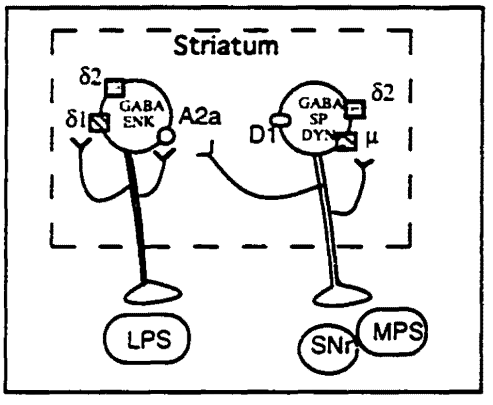


FIGURE 4 - Opioid receptor localization on medium-size spiny neurons in the striatum. Adapted from Noble and Cox, 1995.

6. Serotonin

Serotonin innervates the BG through ascending serotoninergic projections arising from the raphe nuclei. The dorsal ascending pathway, originating from the nucleus raphe dorsalis, projects mainly to the CP with collaterals leading to the NAcc and GP. The

medial ascending pathway, also from the nucleus raphe dorsalis, terminates mainly in the SN, but also sends collaterals to the striatum. The ventral ascending serotoninergic pathway arises from the mesencephalic raphe nuclei and projects to the VTA and the medial forebrain bundle (MFB). From the MFB, 5-HT reaches the amydaloid nuclear complex through the stria terminalis (Steinbusch, 1984).

5-HT mediates both stimulatory and inhibitory actions in the CNS through a broad range of receptor subtypes termed 5-HT_1 (further subtyped B-D) through 5-HT_4 (Julius, 1991). In the striatum, 5-HT inhibits ACh release presumably through 5-HT_2 presynaptic receptors located on cholinergic terminals and cell bodies (Palacios and Dietl, 1988; and Waeber and Palacios, 1994), and GABA release through 5-HT_{1B} receptors located on striatonigral cell bodies (Palacios and Dietl, 1988) and through synaptic contact with the dendritic spines (Soghomoniam et al., 1989). Direct activity of 5-HT on DA neurons in the striatum is controversial. Ennis et al (1981) state that 5-HT inhibitory receptors are located on presynaptic terminals of DA neurons in the striatum, while others, using lesion studies, show evidence that 5-HT binding sites are not associated with dopaminergic cell bodies, dendrites, or terminals in the striatum (Palacios and Dietl, 1988). 5-HT is also thought to synapse with cholinergic terminals in the ventral pallidum. Immunohistochemical studies indicate that ascending serotoninergic neurons innervating the SN primarily terminate in the pars reticulata region (Lovoie and Parent, 1990), and lesion studies indicate that 5-HT synapses with the striatonigral terminals in the SNr which express 5-HT_{1B} receptors (Palacios and Dietl, 1988).

7. Substance P

Substance P is found in medium-size spiny neurons of the striatum (Gerfen, 1992), presumably co-located with GABA and DYN. In the dorsal BG, SP is thought to act specifically on striatal cholinergic interneurons through the SP stimulatory receptor, neurokinin-1 (NK-1) (Gerfen, 1991). Although, immunoreactivity is expressed throughout the striatum, SN, and GP (Haber and Nauta, 1983; and Gerfen, 1991), SP

binding studies have shown dense receptor binding in the striatum and no binding in the SN and only sparse binding in the endopeduncular nucleus, the medial segment of the GP (Danks et al., 1986). This is unusual since these latter two structures are heavily innervated with SP from the striatonigral/endopeduncular projection neurons. In situ hybridization histochemistry has indicated the presence of NK-1 receptor mRNA on large cholinergic neurons in the striatum, however no receptor mRNA is thought to exist on the medium-size spiny neurons in the striatum nor on any neurons in the SN (Gerfen, 1992). SP released from recurrent collaterals of the striatonigral/endopeduncular neurons stimulates ACh release by activating NK-1 receptors located on the large aspiny cholinergic interneurons (Gerfen, 1992). In the ventral striatum, lesion studies have indicated that the NAcc sends SP immunoreactive fibers to the ventral pallidum (Haber and Nauta, 1983) that make synaptic contact on numerous cholinergic neurons (Bolam et al., 1986).

Although SP was shown to specifically interact with cholinergic neurons in the striatum and ventral pallidum (Danks et al., 1986; Gerfen, 1991; and Arenas et al., 1991), it has also been shown to stimulate [^{3}H]-DA release from the cat caudate nucleus and met-ENK from rat striatal tissue slices (Baruch et al., 1988; and Del Río et al., 1983, respectively). Release of [^{3}H]-DA from the caudate was mediated by applying SP directly to the pars compacta of the SN, which is contradictory to Gerfen's (1992) interpretation that nigral neurons do not express SP receptors. However, Baruch et al (1988) suggest that SP-stimulated release of [^{3}H]-DA from the cat caudate nucleus is an indirect effect mediated by a "nigro-thalamo-cortico-striatal loop." Del Rio et al (1983) also suggest that SP innervation of met-ENK containing neurons might be indirect through "an interposed cell"; indeed, because NK-1 receptors are not found on striatal spiny neurons (which contain ENK). SP may be stimulating ACh release which in turn stimulates ENK release by activating M1 muscarinic receptors preferentially located on GABA/ENK neurons in the striatum.

C. Metabolic relationship of glutamate and GABA in the CNS

Glu and GABA are closely related neurotransmitters despite their opposing roles in the CNS. As shown in figure 5, Glu and GABA are very similar in structure and have a close metabolic relationship: Glu is the only immediate precursor of GABA, and GABA is one of many metabolic products of Glu metabolism. Both amino acids are metabolized to various products used in carbohydrate metabolism, and this recycling of metabolites makes it difficult to study Glu and GABA turnover. Glu is metabolized directly from glutamine by glutaminase and from α-ketoglutarate by ornithine aminotransferase. It is also derived from aspartate and α-ketoglutarate through amino-transformation by aspartate transferase, however, which if any of these means of Glu synthesis predominates in glutamatergic neurons has not been fully established. The enzyme responsible for GABA's synthesis, glutamic acid decarboxylase (GAD), is unique in that it serves solely to convert Glu to GABA in GABAergic neurons. This enzyme requires the coenzyme pyridoxal phosphate, and its activity is regulated by both Glu and adenosine analogs (ATP). High levels of Glu and ATP reduce GABA synthesis by preventing proper GAD-coenzyme association (Cooper et al., 1991). GABA transaminase (GABA-T), the enzyme which initiates the primary pathway of GABA metabolism (Lippert et al., 1977), also requires pyridoxal phosphate and is more widely distributed in the body than GAD.

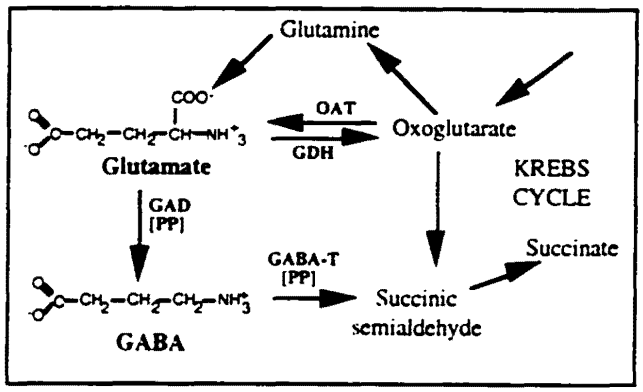


FIGURE 5 - Metabolic relationship of Glutamate and GABA. Abbreviations: GAD, Glutamic acid decarboxylase; PP, pyridoxyl phosphate; GABA-T, GABA transaminase; GDH, glutamate dehydrogenase; OAT, ornithine aminotransferase.

Inhibition of GABA metabolism by inhibiting GABA-T activity has been extensively studied in *in vitro* release experiments as well as clinically as an anticonvulsant. Enzyme

inhibitors such as hydrazides, amino-oxyacidic acid (AOAA), gabaculine, γ-acetylenic GABA, and γ-vinyl GABA (vigabatrin) effectively block GABA-T, but they also show varying degrees of blocking GABA uptake, GABA receptor binding, and GABA synthesis through inhibition of GAD activity (Loscher, 1980). Gabaculine and AOAA, for instance, are the most potent inhibitors of GABA-T, but gabaculine is also a potent inhibitor of uptake, and AOAA shows equipotency at inhibiting GAD activity (Loscher, 1980). Vigabatrin, thought to be one of the most selective inhibitors of GABA metabolism, was found to increase extracellular GABA concentrations in rats primarily through blockade of GABA uptake and not through inhibition of metabolism (Jolkkonen et al., 1992). While preventing GABA metabolism will theoretically increase extracellular GABA, blocking Glu metabolism may ultimately decrease GABA content through either direct enzyme inhibition of GAD or through inhibition of GAD-pyridoxal phosphate formation by excess Glu.

D. Pharmacological significance of the glutamatergic and GABAergic synapse in the striatum

GABA activity is thought to be regulated at the synaptic level by both a presynaptic $GABA_B$ receptor and a reuptake transporter. The $GABA_B$ receptor was first discovered in 1981 by Hill and Bowery (1981) to be a novel bicuculline-insensitive GABA binding site that specifically binds baclofen. It was pharmacologically distinguished from the classical $GABA_A$ receptor, but evidence that it acted as an autoreceptor in the CNS was described later when Anderson and Mitchell (1985) showed that baclofen selectively inhibited [^{3}H]-GABA release from the rat median eminence. Through the autoreceptor, baclofen the classical $GABA_B$ receptor agonist, is thought to inhibit GABA release in many brain areas, but in a frequency-dependent manner. Generally, under high stimulated GABA release baclofen has little inhibitory effect, but at basal and low stimulus levels of either 0.5 Hz or 15 mM potassium, baclofen inhibits endogenous GABA release from rat striatum and cortical slices by as much as 60-70 percent (Waldmeier et al., 1988; and Waldmeier and

Baumann, 1990). The extent to which autoreceptors regulate GABA activity within the striatum has not been firmly established, probably because GABA is released from several sites including recurrent collaterals, dendritic spines and interneurons, and it is not known which population of neurons contains $GABA_B$ receptors. For instance, autoreceptor regulation of GABA release is thought to be only on a subpopulation of GABA neurons in the striatum and not on the terminals of recurrent collaterals (Seabrook et al., 1991), because the cell bodies of these neurons, as well as the nigral projection neurons, do not appear to possess functional $GABA_B$ receptors (Waldmeier et al., 1989).

Synaptic GABA uptake is thought to be an important recognition site involved in terminating GABA transmission (Schousboe et al., 1979), and specific uptake transporter inhibitors have been studied as a means of increasing synaptic GABA in *in vitro* and *in vivo* experiments and as potential anticonvulsants. Nipecotic acid and its derivatives are among the most potent inhibitors of GABA transport (Schousboe et al., 1979), but specificity lacks because most derivatives are unable to distinguish the neuronal transporter from the glial transporter (Schousboe et al., 1979; and Larsson et al., 1988), and nipecotic acid may possess substrate affinity for the transporter and displace GABA from intra-neuronal stores (Johnston et al., 1976; and Waldmeier et al., 1992). SK&F 89976, a diphenyl-3-butenyl derivative of nipecotic acid, is more potent than nipecotic acid and does not possess transporter substrate activity (Waldmeier et al., 1992). It shows no activity on GABA receptors or GABA metabolism, and its lipophilic moiety allows it to readily cross the blood brain barrier. However, like nipecotic acid, SK&F 89976 blocks both neuronal and glial GABA uptake (Yunger et al., 1984).

Glutamatergic transmission is controlled at its synapse by a high affinity transporter which terminates Glu transmission by transporting Glu from the synapse into either the presynaptic terminal or into glial cells which surround the terminal (Robinson et al., 1991). Various analogs of Glu and kainic acid are known to inhibit Glu transport with varying degrees of potency and selectivity. Threo-3-hydroxyaspartate (THA) is among the most

potent of uptake blockers (Johnston et al., 1979) but possesses significant activity on NMDA receptors (Olferman et al., 1988). Dihydrokainic acid, whose effects were recently studied on *in vivo* Glu release (Fallgren and Paulsen, 1996), is primarily devoid of Glu receptor activity, but is a much weaker blocker of Glu reuptake (Johnston, et al., 1979). L-4-*trans*-pyrollidine-2,4-dicarboxylic acid (L-trans-2,4-PDC) is the most recently discovered selective inhibitor of Glu uptake. It competitively inhibits Glu uptake with a K_i of 4.6 μM and is considered one of the most potent and preferred inhibitors of the Glu synaptic transporter (Bridges et al., 1991, Kanai et al., 1994). Waldmeier et al (1993) have shown the effects of L-trans-2,4-PDC on Glu release from rat cortex tissue. They show that potassium stimulated release of Glu from synaptosomes is decreased significantly by 10 to 300 μM L-trans-2,4-PDC, while basal release is increased 3 to 4 fold under similar concentrations of L-trans-2,4-PDC. They, as well as others (Kanai et al., 1994), believe that L-trans-2,4-PDC can serve as a substrate for the transporter and displace Glu from its terminal storage sites.

Autoreceptor regulation of Glu release is of some controversy probably because Glu, being an excitatory amino acid (EAA), is historically not known to act on an inhibitory receptor. Pharmacological studies indicate that NMDA receptors are on Glu terminals and mediate an apparent "positive feedback" effect (Errington et al., 1987; and Young and Bradford, 1991), although binding studies in lesioned animals suggests that no presynaptic receptor is present (Greenamyre and Young, 1989). Recent studies (Chittajallu et al., 1996) propose that kainate receptors (non-AMPA) are located presynaptically on Glu terminals of the rat hippocampus and mediate an inhibitory role on Glu release, while earlier reports (Butcher et al., 1987) suggested that kainic acid mediates an *in vivo* release of Glu from the rat striatum, independent of Ca^{2+}.

E. Stimulators of neurotransmitter release

Depolarizing stimuli such as elevated levels of potassium, excitatory amino acid receptor agonists and 4-aminopyridine stimulate release of neurotransmitters from interneuronal stores presumably by inducing an influx of calcium into to the neuron through voltage-sensitive Ca^{2+} channels. Potassium stimulates release of Glu from rat cortical synaptosomes (Waldmeier et al., 1993), *in vivo* Glu and GABA release from rat hippocampus (Rowley et al., 1995) and GABA release from rat NAcc (Smith and Sharp, 1994b), and this release is mediated by potassium induced changes in the neuronal membrane potential. Elevated extracellular potassium weakens the potassium concentration gradient that normally moves potassium out of the neuron; this causes an unbalanced electrical potassium gradient which accelerates potassium influx into the neuron. If nicotinic-ACh receptors are present on these neurons, it is anticipated that their activation will induce depolarization through an influx of Na^+ ions. Depolarization of the neuron, arising from an intracellular increase in positive charge, opens voltage-sensitive Ca^{2+} channels which enables an influx of Ca^{2+}.

The NMDA and AMPA excitatory amino acid receptors both regulate a channel which is permeable to Na^+ and potassium, but the NMDA receptor channel is also highly permeable to Ca^{2+} and is physiologically blocked by the presence of Mg^{2+}. The Mg^{2+} blockade is voltage dependent which means the blockade can be relieved by a depolarization of the neuron by at least 20 to 30 millivolts. The influx of Ca^{2+} through the NMDA receptor channel may serve directly to mediate Ca^{2+}-dependent neurotransmitter release by exocytosis and/or as a carrier of positive charge into the neuron which depolarizes the neuron and ultimately opens voltage-sensitive Ca^{2+} channels (Kandel and Schwartz, 1991). The most significant aspect of opening the NMDA receptor channel is that it is fairly slow and maintains a long-lasting influx of Ca^{2+}. On the other hand, the AMPA receptor mediates a fast and brief influx of Na^+ which evokes a "transient depolarization" that opens voltage-sensitive Ca^{2+} channels (Kötter, 1994). Ultimately,

both NMDA and AMPA receptor activation lead to an influx of Ca^{2+} which may induce neurotransmitter release from neurons on which the receptors are located.

4-aminopyridine (4-AP) is a blocker of the voltage-dependent potassium channel (Albuquerque et al., 1988) and can evoke transmitter release by blocking the channel and subsequently enhancing inward Ca^{2+} currents (Thesleff, 1980). During a neuron's resting state, potassium channels are normally open and the membrane is hyperpolarized; by blocking these channels, the membrane potential becomes more positive and closer to the threshold for neuron depolarization (Kandel and Schwartz, 1991). 4-AP may therefore not evoke "resting" transmitter release and require a small stimulus for depolarization (Thesleff, 1980), however, this is disputed by Hu and Fredholm (1991) who showed 4-AP induced release of [^{3}H]-noradrenaline in a concentration-dependent manner from rat hippocampal slices without field stimulation.

F. Specific calcium channels involved in axonal and dendritic neurotransmitter release

Exocytotic release of neurotransmitters is mediated by Ca^{2+} entry into presynaptic nerve terminals through voltage-sensitive Ca^{2+} channels (Kandel, 1991). Central neurons contain several types of Ca^{2+} channels which can be pharmacologically defined by specific channel antagonists, and these antagonists have been used to explore the involvement of various Ca^{2+} channels on transmitter release. Immunohistochemical studies have indicated that L-type Ca^{2+} channels exists specifically on cell bodies and proximal dendrites of central neurons (Westenbroek et al., 1992; and Hell et al., 1993), while N- and P-type channels are located on the soma, axon terminals, and the entire length of the dendrites (Hillman et al., 1991; and Westenbroek et al., 1992). The L-type channel blocker, nifedipine, and the N- and P- type blockers ω-conotoxin GVIA (CnTx GVIA) and ω-agatoxin IVa (AgTx IVa), respectively, have been commonly used in identifying specific Ca^{2+} channels involved in Ca^{2+}-dependent transmitter release.

Previous reports have shown that potassium stimulated release of [^{3}H]-Glu and [^{3}H]-DA from rat striatal synaptosomes (ie. from axon terminals) was partially reduced by the P-type Ca^{2+} channel blocker and not affected by the N-type channel blocker (Turner et al., 1993), while release of [^{3}H]-GABA was only sensitive to N-type Ca^{2+} channel blockade (Lecharny et al., 1995). Discrepancies, however, can exist between results obtained with intact synapses and those using synaptosomal preparations, and preloaded [^{3}H]-transmitter release may differ from release of endogenous transmitters (Herdon et al., 1985; Nicholls and Sihra, 1986; and Burke et al., 1993). Endogenous Glu release from striatal slices was reported to be highly sensitive to AgTx IVa, slightly sensitive to CnTx GVIA, but insensitive to nifedipine (Kimura et al., 1995). Burke et al (1993) reported that Glu release from rat hippocampal slices was primarily dependent on Ca^{2+} entry through the P-type channel and to a lesser extent, the N-type, however both P- and N-type channels were involved in GABA release. Takahashi and Momiyama (1993) have also presented similar evidence with selective Ca^{2+} channel blockers on excitatory postsynaptic currents in rat hippocampus and inhibitory GABAergic currents in rat cerebellar and spinal neurons.

The selective localization of the L-type channel on the soma and dendrites of central neurons may indicate the channel's role in cellular functions such as gene expression and enzyme activity (Hell et al., 1993). The channel is also thought to be important in mediating transmitter release from dendrites (Simmons et al., 1995), and release studies in the presence of nifedipine may be a useful tool in identifying dendritic release processes. Simmons et al (1995) have shown that dendritic DYN release from rat hippocampus was sensitive to both L- and N-type Ca^{2+} channel antagonists, while N-type but not L-type channel antagonists inhibited axonal release. GABA release in the striatum is both axonal and dendritic and is therefore likely to be dependent on Ca^{2+} influx through L-, P- and N-type Ca^{2+} channels.

G. Dopamine and opiate interactions in primary cultures of striatal neurons

Functional experiments such as transmitter release and cyclase assays with primary striatal cultures can be distinctly different from other *in vitro* striatal preparations such as synaptosomes and tissue slices: although primary cultures express an abundance of diverse transmitter receptors, they consist of only inherent striatal neurons (primarily GABAergic) and no efferent neurons such as Glu, DA and serotonin, like seen in other striatal preparations. This distinction can enable more direct effects on striatal neurons to be analyzed, and less influence from simultaneous efferent transmitter activity. Receptor expression in striatal cultures also may vary from expression in adult rat striatum, primarily because of embryonic development of striatal receptors. Pharmacological evidence suggests that both D_1 and D_2 DA receptors are expressed in primary striatal cultures derived from fetal rat (Eriksson et al., 1991; and Hoyt and Reynolds, 1996) and fetal mouse (Chneiweiss et al., 1988; Kowalski and Giraud, 1993; and Maus et al., 1993); μ-opiate receptors, as well, are thought to be expressed in primary fetal striatal cultures (McDowell and Kitchen, 1987; Chneiweiss et al., 1988; and Eriksson et al., 1991). Opiate receptor binding studies in rat brain have shown that ontogenesis of δ-opiate receptors was not detected before postnatal day 10 (McDowell and Kitchen, 1986 and 1987), and δ-type receptors appear later than the μ-type during ontogeny (McDowell and Kitchen, 1986 and 1987; and Eriksson et al., 1991). This would suggest a lack of δ-type opiate receptors in primary cultures derived from fetal rat brain. However, reports by at least two separate groups showed functional evidence, through adenylyl cyclase assays, that δ-type opiate receptors are located in primary striatal cultures and are most likely coupled to μ-type receptors (Chneiweiss et al., 1988; and Eriksson et al., 1991). Chneiweiss et al (1988) also presented evidence suggesting that these opiate receptors are located on primary striatal cultured neurons possessing D_1 DA receptors (this was previously discussed in the *opiate peptides* section of the *Introduction* as it pertains to the adult rat striatum).

Stimulated release GABA from primary striatal cultures is elicited by elevated potassium, veratridine, and EAA receptor agonists Glu, aspartate, NMDA, AMPA, kainate and quisqualate (Weiss, 1988a,b; Pin et al., 1989; and Pin and Bockaert, 1989). Little evidence exists that shows GABA release is evoked or inhibited by DA or opiate receptor stimulation. Previous reports have shown that both endogenous (Pin and Bockaert, 1987 and 1989) and [^{3}H]-GABA (Weiss, 1988a and 1990) release from primary striatal cultures induced by potassium was Ca^{2+}-dependent and release elicited by EAAs was not. Potassium stimulated release was believed to have occurred through exocytosis, and release stimulated by Glu was thought to result from reversal of the synaptic GABA transporter. Transporter reversal was postulated because release stimulated by EAAs was blocked by nipecotic acid, while potassium depolarization still induced GABA release under identical conditions (Pin and Bockaert, 1989). EAA stimulated release of GABA is also thought to involve a depolarization-dependent reversal of the GABA transporter which is highly dependent on Na^+. An intracellular accumulation of Na^+, which follows EAA receptor stimulation, rapidly reverses the Na^+ gradient required of normal GABA uptake; Na^+ (and Cl^-) is instead pumped out of the neuron with GABA (Weiss, 1988a; and Pin and Bockaert, 1989).

H. HPLC analysis of amino acids in biological tissue

Various techniques have been explored for the analysis of amino acids in biological tissue (reviewed by Reynolds and Pearson, 1993), and a commonly used method which is relatively simple and convenient, employs the versatility of reverse-phase high performance liquid chromatography (HPLC) coupled with the sensitivity of electrochemical detection. Isocratic separation of reverse-phase HPLC is based on the hydrophobicity of the amino acids; Glu being fairly hydrophilic compared to GABA. Samples injected into the HPLC pass through a reverse phase column made of carbon-chain resin which separates the contents of the samples primarily through hydrophobic interactions between the sample and

the resin. The more hydrophobic the passing compound, the longer it remains on the column and the later it elutes. Other parameters also influence column separation: the concentration of organic modifier in the mobile phase (ie. methanol or acetonitrile) and the mobile phase pH have significant roles on the separation and elution of the amino acids. The larger the concentration of methanol in the mobile phase the quicker amino acids move through the column, while the more acidic the pH, the longer the amino acids remain on the column, and their column elution time is increased. By varying the separation and elution parameters, optimal separation of the amino acids can be obtained.

Electrochemical detection is used to measure the amino acids as they elute off the column. Unlike the catecholamines, Glu and GABA are not electroactive within the parameters for this type of detection, so they must be derivatized prior to analysis. In this study, the amino acids were derivatized with an o-phthaladehyde-sulphite (OPA-SO_3) substituent on the amino acid primary amine which renders them electroactive through the sulphite ion (Jacobs, 1987). The reaction is depicted below in figure 6. This method, originally described by Jacobs (1987), is a modification of an original amino acid derivatization assay developed for fluorimetric detection (Lindroth and Mopper, 1979) and later for electrochemical detection (Joseph and Davies, 1983). Pearson et al (1991) as well as others have taken Jacobs' methodology and designed specific parameters for measuring Glu and GABA content in postmortem Huntington's disease brain tissue, GABA release from rat NAcc (Smith and Sharp, 1994a,b), and GABA release from rat hippocampus (Rowley et al., 1995). The precise method of amino acid measurement used in my study was based on both Pearson et al and Smith and Sharp publications, but was

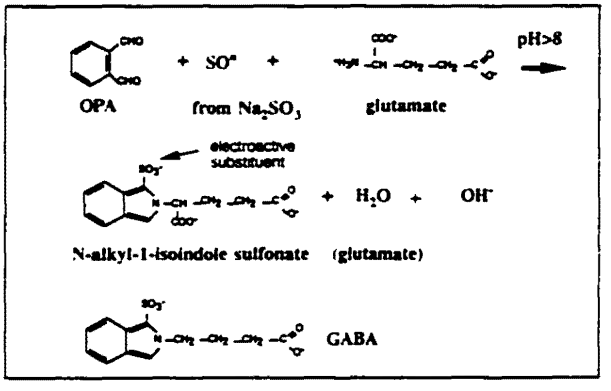


FIGURE 6 - Schematic representation of OPA-SO_3 derivatization of glutamate and GABA. Adapted from Jacobs, 1987.

improved to allow better separation and resolution of Glu and GABA (Phillips and Cox, 1997; and discussed later).

I. Basic hypothesis and specific objectives

I have studied Glu and GABA release from *in vitro* preparations of the rat striatum to evaluate characteristic influences involved in the release of the amino acids. Both neuron localization and specific receptor expression suggests that the amino acid release is regulated by DA, ACh and opiate peptides, as well as other transmitters. I propose that the release of Glu and GABA is regulated by autoreceptor activity and presynaptic transporter reuptake, and that this release is under significant regulatory control by local striatal transmitters. DA is likely to exert a facilitory influence on GABA release through D_1 DA receptors, while μ-opiate receptor stimulation is likely to yield an inhibitory influence.

Amino acid release is studied from striatal tissue slices and fetal rat primary striatal cultures with the major difference in the two being only GABA, and not Glu, is released from the cultures. GABA release from the cultures allows a more focused approach to evaluating striatal GABA release, because there is no endogenous release of DA or Glu which may influence the depolarization-evoked release of GABA.

The primary objectives of this project are: (1) develop a HPLC/electrochemical assay with sufficient sensitivity to measure reliably the amounts of endogenous Glu and GABA released from *in vitro* tissue preparations under basal and depolarizing conditions; (2) obtain selective release stimuli of Glu and GABA and determine the origins of the stimulated amino acid release (axonal and/or dendritic) from striatal tissue slices using selective P-, N- and L- type Ca^{2+} channel antagonists; (3) investigate the striatal transmitters which influence Glu and GABA release under depolarizing conditions; and (4) evaluate the autoreceptor regulation of GABA release, as well as DA and μ-opiate receptor influences on GABA release, from fetal rat primary striatal cultures.

METHODS

A. Assay for glutamate and GABA release from rat caudate-putamen tissue slices

A novel microtiter plate release assay was employed to study the release of the amino acids from rat CP tissue slices. Male Sprague Dawley rats (200-300 g) were euthanatized by decapitation, and the CP immediately dissected from the brain and chopped into 225-μm slices; the chopped tissue was rotated through 90 degrees and chopped again. (Slices chopped in two dimensions may be referred to as tissue "prisms"; however, for consistency with other literature reports I will refer to the assayed tissue preparation as slices.) The tissue slices were rinsed three times in oxygenated modified Krebs-HEPES buffer (MKB, in mM: 127 NaCl, 5 KCl, 1.3 NaH_2PO_4, 1.2 $MgSO_4$, 2.5 $CaCl_2$, 10 glucose, 15 HEPES, adjusted to pH 7.4), then placed in a final suspension of MKB to yield approximately 7.5-10 mg tissue/ml MKB. 200 μl aliquots of the tissue suspension (1.5 to 2 mg of tissue) were placed into individual wells of a modified 96-well microtiter plate (Millipore Multiscreen, Millipore Corp., Bedford, MA). The plate contains a 0.65-μm hydrophilic PVDF membrane on the bottom of each well. Under aspiration, buffer passes through the membrane and tissue is retained in the well. The microtiter plate with tissue underwent three separate 5 minute incubations at 37° Celsius in 250 μl of oxygenated incubation media per well. First incubations contained MKB and receptor antagonists. Second incubations were in MKB and drug contents identical to the first incubation plus receptor agonists where appropriate. The third and final incubations contained contents identical to the previous two incubations plus the releasing stimulus: as discussed later, elevated KCl (20 to 100 mM) with NaCl reduced proportionally to maintain constant ionic strength, 4-AP and NMDA. In other wells, MKB was used in the final incubation to assay basal release. In general, agonists were added to the second and third incubations and antagonist were added to all three incubations when receptor-specific agonist-induced activity was assayed. At the end of each 5 minute incubation period, the microtiter plate

was removed from the incubator and the releasate from the individual wells, about 250 µl, was filtered under mild vacuum into corresponding wells of a second 96-well plate (a normal 96-well plate without membrane filters). Releasate from each of the three incubations was collected into separate plates; 100 µl of each releasate sample from the final incubation were used for HPLC analysis of Glu and GABA as described later. Tissue in each well was analyzed for total protein content using a simplified method described by Peterson (1977). All chemicals were from Sigma Chemical Company (St. Louis, MO), and drug receptor ligands were purchased from Research Biochemicals International (Natick, MA), unless otherwise noted. ω-Agatoxin IVa, the P-type Ca^{2+} channel blocker, was a gift from Pfizer Inc. (Groton, CT).

B. Fetal rat primary striatal cultures

The following two media were used in culturing the striatal neurons:

NuSerum containing media (NS-media)	B-27 containing media (B27-media)
Neurobasal medium †	Neurobasal medium †
20 µM glutamate	20 µM glutamate
500 µM glutamine	500 µM glutamine
10% NuSerum IV *	2% B-27 supplement ††
1% penicillin-streptomycin	1% penicillin-streptomycin
16.6 mM glucose	16.6 mM glucose

Media were filtered through a 0.2 µm filter into a sterile container. All above products were purchased from Gibco BRL (Gaithersburg, MD) except * purchased from Collaborative Biomedical Products (Bedford, MA). †, Neurobasal medium contains inorganic salts, amino acids and vitamins. ††, B-27 supplement primarily contains steroids, antioxidants and proteins. See Brewer et al (1993) for detailed composition.

1. Plate preparation

Sterile six-well plates were pretreated with 2 ml per well of 1:500 polyethylenimine (PEI) in 50 mM sodium borate pH 7.4 overnight, then washed with PBS pH 7.4 twice before 2 ml of NS-media were added to each well. The plate pretreated with media was allowed to incubate as before for 4 to 24 hours prior to initiation of the culture; incubations occurred at 37° Celsius in 95% oxygen and 5% CO_2.

2. Fetal striatum dissection and neuron culture preparation

Female Sprague-Dawley rats, 18-day pregnant, under 2.5% halothane anesthesia, were euthanatized by decapitation. The fetuses were removed from the mother and placed into sterile 0.9% saline. Heads were removed from the fetuses and soaked in sterile 70% EtOH for approximately 30 seconds before being washed twice with saline. Heads remained on ice in a petri dish of saline prior to dissection. The striata were dissected out in a petri dish, bathed in Hanks' balanced salt solution with 20 mM HEPES, pH 7.4 (1xHBSS from 10xHBSS in g/L: 1.4 $CaCl_2$, 4.0 KCl, 0.6 KH_2P0_4, 1.0 $MgCl_2$, 1.0 $MgSO_2$, 80 NaCl, 0.9 Na_2HPO_4, 10 glucose; Gibco BRL, Gaithersburg, MD) and stored on ice in 1xHBSS. When dissection was completed, 1xHBSS was removed by aspiration and the tissue was suspended in 2 ml of NS-media; this and the following procedures were conducted under a tissue culture hood. The tissue was triturated into suspension with a fire-narrowed Pasteur pipette and resuspended in 10 ml of NS-media. Cells were counted from an aliquot of the 10 ml suspension using a Coulter Counter ZM (Coulter Electronics, Inc., Hialeah, FL). The tissue suspension was diluted with fresh NS-media at 37° Celsius to yield approximately $1x10^6$ cells/ml. After the NS-Media was removed from the pretreated plates, 2 mls of the final tissue suspension were plated into each well to yield $2x10^6$ cells per well.

After one day of incubation in 95% oxygen and 5% CO_2, NS-media was removed from the plates, and 2 ml of B27-media were added to each well. Serum-free B-27 supplement (with reductions in glutamine concentrations) were important in reducing glial growth in the cultures (Brewer et al., 1993). The primary striatal cultures (PSC) were grown for 7 days at 37° Celsius in 95% oxygen and 5% CO_2.

C. Assay for GABA release from fetal rat primary striatal cultures

GABA release experiments were conducted in the six-well culture dishes using incubation steps similar to the tissue slice release assay described previously. Following the seventh day of incubation, the plates were removed from the incubator and the release assay was conducted on the bench. B27-media contained in each well was removed by aspiration and each well was washed twice with one ml of MKB. The cultured neurons underwent three separate 5 minute incubations at 37° Celsius in 0.75 ml of oxygenated incubation media per well. An "acid wash" of the PSC was conducted in a fourth and final incubation at 37° Celsius for 5 minutes using 0.75 ml of 0.2 N HCl per well. This released a "total amount" of GABA present in each well and allowed for release to be expressed as percent fractional release. GABA content from the second, third and fourth incubations was assayed by HPLC and electrochemical detection; prior to analysis of acid wash samples, 31.5 µl of 5 N NaOH were added to each of the 0.75 ml of acid wash samples to enable a more optimal sample pH for the derivatization process as described below.

D. Measurement of glutamate and GABA through HPLC analysis

Samples (standards or releasate, but not PSC acid wash samples) containing Glu and/or GABA were derivatized by combining 100 µl of sample with 20 µl of internal standard (3.3 µM homoglutamine) and 12 µl of OPA-SO_3 solution (22 mg OPA, 0.5 ml 0.0313 M Na_2SO_3, 0.5 ml EtOH, and 9 ml 0.1 M Borax). Because acid wash from PSC release experiments yielded large amounts of GABA, these samples were first diluted 1:7 with H_2O (12.5 µl of acid wash sample plus 87.5 µl of H_2O for a 100 µl sample). Samples, internal standard and OPA-SO_3 reagent were individually filtered through a 0.22-µm syringe filter prior to mixing. The mixture was allowed to react for 15 minutes at 37° Celsius before 20 µl of the reaction mixture was injected onto the column. An ESA model 580 solvent delivery module (ESA, Inc., Chelmsford, MA) pumped the mobile phase (0.1 M Na_2HPO_4, 25 µM EDTA, 5% MeOH, adjusted to pH 5.18 with NaOH) at a consistent

flow rate of 1.2 ml/minute. During method development the mobile phase pH ranged from 5.18 to 5.60. Samples were injected into a 20 µl injection loop (model 7125, Rheodyne, Inc., Cotati, CA) which preceded the guard column (5 µm-C18 Microsorb, Rainin Instrument Co., Inc., Emeryville, CA) and reverse phase analytical column (3 µm-C18, 4.6 x 80 mm HR-80, ESA Inc.). An ESA model 5100A Coulochem detector with guard cell and 5011 dual analytical cell were used for the electrochemical detection. In preliminary experiments it was determined that optimal detector potentials were 650 mV for the guard cell and 350 mV and 600 mV for the D1 and D2 electrodes of the dual analytical cell, respectively. The derivatized amino acids were not affected by the screening 350 mV potential but responded to the second potential of 600 mV by a shift in electrons of the sulphite substituent. This induced a voltage response recorded in microvolts per second which is reported as Area Under the Curve (AUC) for the peak.

After preliminary studies with internal standards used in previously published work, a novel internal standard, homoglutamine (HGLN), was used routinely in quantifying the amounts of amino acids in the tissue releasate (see Results). Standard curves were first established for LCED determination of derivatized Glu and GABA by injecting standards of Glu and GABA in increasing amounts with a consistent amount (10 pmol) of derivatized HGLN. Each injection generated a ratio of AUC of the Glu and GABA peak to the AUC of the 10 pmol HGLN peak. The ratio increased as the amounts of Glu and GABA increased. Glu and GABA standards were diluted to appropriate concentrations from 10 mM stock solutions to yield 1 to 50 pmol amounts in the injected samples. HGLN was also made from a 10 mM stock solution to yield a final amount of 10 pmol in the injected sample. Glu stocks were made in 25% methanol and GABA and HGLN stocks were made in 50% methanol and remained stable for 2 months when stored at 4° Celsius. To determine the amounts of Glu and GABA in the tissue releasates, a ratio of the AUC for the sample peaks of Glu and GABA to the AUC of 10 pmol HGLN in each sample injection

was calculated. This ratio was compared to the AUC ratios of the standard curves, and Glu and GABA content was determined.

LCED analysis of amino acid standards that were not derivatized (contained no OPA-SO_3 reagent) yielded chromatograms with no discernible peaks. Blank samples containing OPA-SO_3 reagent with MKB and no amino acid standards also yielded chromatograms with no discernible peaks which would interfere with the amino acid analysis; only a characteristic solvent front was evident.

E. Statistical analysis

Analysis was conducted on experiments performed in triplicate (or greater) for agonist- and antagonist- induced effects. Analysis of variance (ANOVA) with Scheffe post test was used for analysis of tissue slice release data. *P* values were computed from raw data which were expressed in [pmol amino acid release per mg protein]. Release of GABA from primary striatal cultures was analyzed by paired *t*-test. The total amount of GABA and the amount of GABA released per tissue culture well varied significantly between experiments (ie. different neural preparations) but were relatively consistent within each preparation. Treatments were compared within each culture by paired *t*-test using estimates of fractional release (amount released as percent of total GABA present in the well). Treatments were considered to yield significant effects when $P<0.05$.

RESULTS

A. Development of a sensitive HPLC assay to reliably measure glutamate and GABA release

As discussed earlier, reverse phase HPLC with electrochemical detection of pre-column OPA-SO_3 amino acid derivatives has recently been utilized to measure *in vitro* levels of Glu and GABA (Pearson et al., 1991) and *in vivo* levels of GABA (Smith and Sharp, 1994a,b) in brain tissue. The method I used to assay Glu and GABA release was derived from the above protocols and was discussed in detail in the *Methods* section. However to achieve greater reliability and chromatogram resolution, several steps were undertaken to improve the published methods.

1. Internal standard selection

Amino acids homoserine (HSER), β-aminobutyric acid (BABA) and homoglutamine (HGLN) were used as potential internal standards. HSER is an endogenous amino acid intermediate used by Donzanti and Yamamoto (1988) as an internal standard in an OPA-β-mercaptoethanol method of derivatizing amino acids for HPLC analysis. BABA, an amino acid similar to GABA, was used by Pearson et al (1991) as an internal standard used in the LCED analysis of the OPA-SO_3 derivatized amino acids from brain tissue homogenates. Each internal standard (20 µl) was derivatized separately with 50 µl of Glu and 50 µl of GABA solutions, and 12 µl of OPA-SO_3 derivatization solution (the stock concentration of Na_2SO_3 was 1.0 M). 20 µl of the total 132 µl in the derivatization reaction were injected into the HPLC. The chromatograms in figure 7 show the elution profiles of HSER and BABA (10 pmol) together with Glu and GABA standards (10 pmol each). HSER co-eluted with extraneous peaks under the current chromatographic conditions (figure 7A), and BABA was shown to elute after GABA, as was also shown by Pearson et al (1991), with much less detector response (figure 7B). The elution profile of HGLN, a non-

endogenous amino acid, is shown in figure 8. It yielded a good detector response and eluted between Glu and GABA; it was also free of any apparent co-eluates.

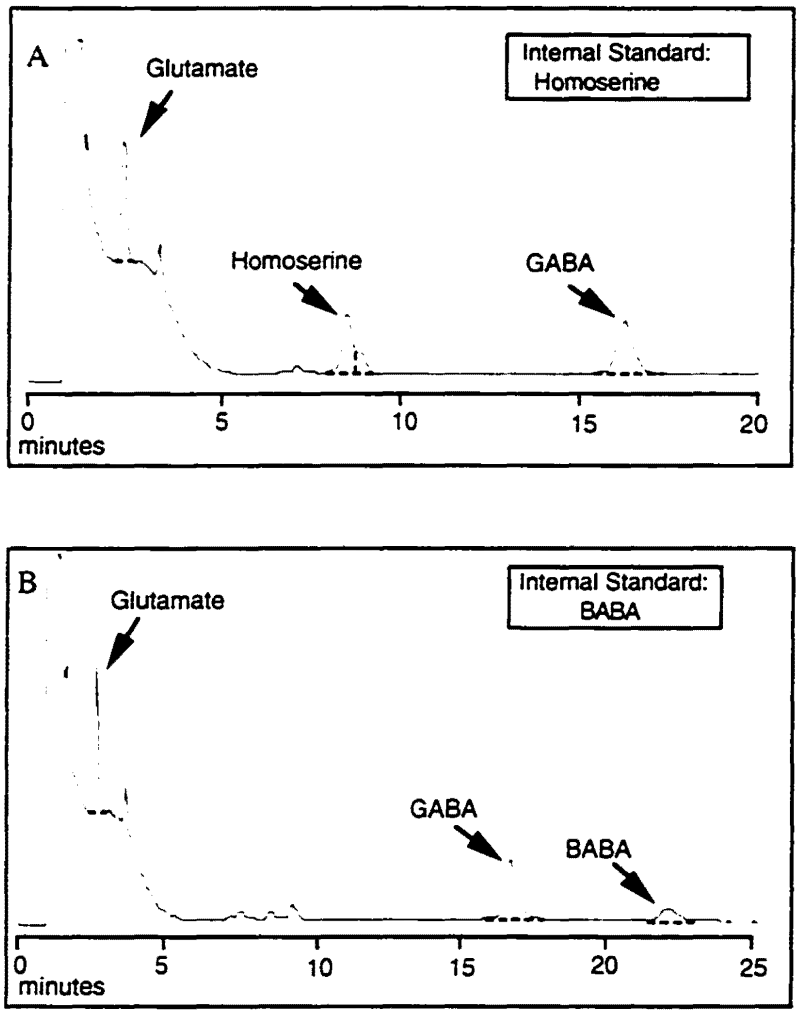


FIGURE 7 - Chromatograms of OPA-SO_3 derivatives of amino acids showing the elution positions of internal standards. Internal standards, HSER and BABA, were derivatized separately with Glu and GABA and 20 µl (10 pmol each amino acid) were injected into the HPLC as described in the text. In A, homoserine is shown co-eluting with extraneous OPA reagent, and as indicated in B, BABA eluted after GABA and yielded a weak detector response. The eluant pH of both chromatograms was 5.60 and the flow rate was 1.0 ml/minute.

2. Baseline elution of glutamate and GABA

The chromatograms in figure 7 above, as well as in previous reported work by Pearson et al (1991) and Rowley et al (1995) show the derivatized Glu product eluting off of the shoulder of an early eluting front. This large front is most likely a result of excess sodium sulphite in the OPA-SO_3 derivatization solution. The sulphite is the electroactive element of the derivatized amino acids (Jacobs, 1987), and large amounts produce high

current interference which compromise baseline elution of Glu. Figure 8 represents chromatograms of amino acid standards derivatized in an OPA-SO_3 solution containing two different concentrations of sulphite: final concentrations of 50 mM (figure 8A) and 1.56 mM (figure 8B) sodium sulphite. The lower amount of sulphite enabled the derivatized Glu product to elute at the baseline level of the chromatogram without affecting its detector response (compare figures 8A and 8B).

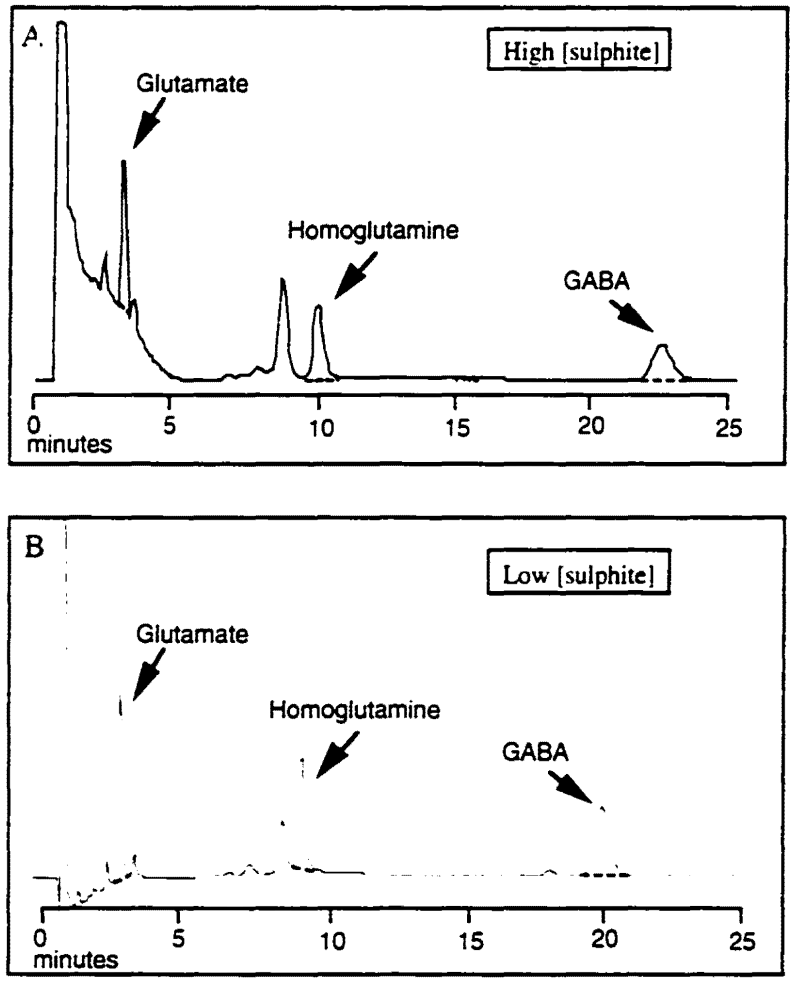


FIGURE 8 - Chromatograms of amino acids derivatized with OPA under different concentrations of sodium sulphite. A: Glu, HGLN and GABA standards were derivatized with OPA solution containing 50 mM sodium sulphite as described in the text and 20 µl were injected into the HPLC. Excessive amounts of sulphite were injected with the amino acid samples (10 pmol) which caused an initial interference with the baseline elution of Glu. B: Amino acids were derivatized with OPA solution containing 1.56 mM sodium sulphite and a 20 µl sample was injected into the HPLC. The initial reagent interference is removed by the reduction in sulphite used in the derivatization solution; this allowed the detector response to return to the baseline level prior to the Glu elution. The mobile phase pH used in both chromatographic analyses was 5.41 and the flow rate was 1.0 ml/minute.

The effect of excess sulphite on Glu detection is reflected in the standard curves depicted in figure 9 which were generated from injections of Glu and GABA standards with 10 pmol HGLN internal standard. When derivatized with the larger amount of

Na_2SO_3 in figure 9A, detection of high amounts of Glu was impaired because it eluted on the shoulder of the interfering reaction products. This caused non-linearity in the standard curve for Glu at Glu amounts above 25 pmol. GABA and HGLN responses were not affected. However, with less sulphite used in the derivatization solution, baseline elution of Glu yielded a consistent detector response with a near linear relationship between Glu and the internal standard response up to 50 pmol Glu (figure 9B). Standard curves for the LCED analysis of Glu and GABA were determined in order to quantify unknown amounts of Glu and GABA released from the tissue slice assay.

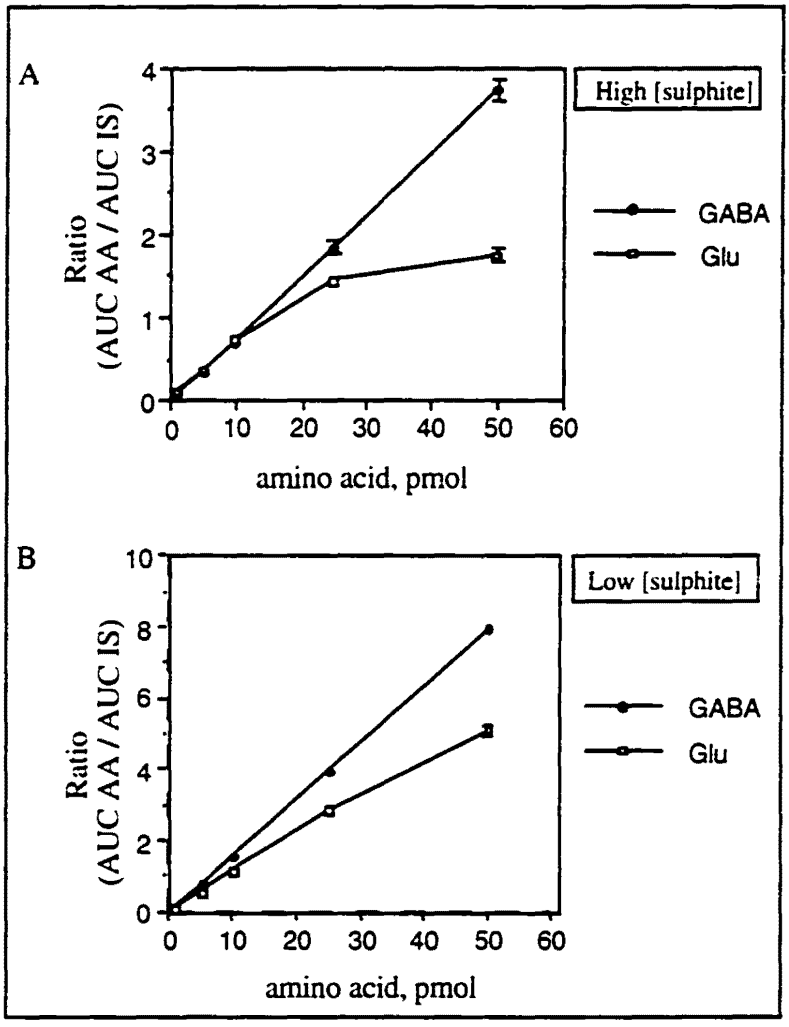
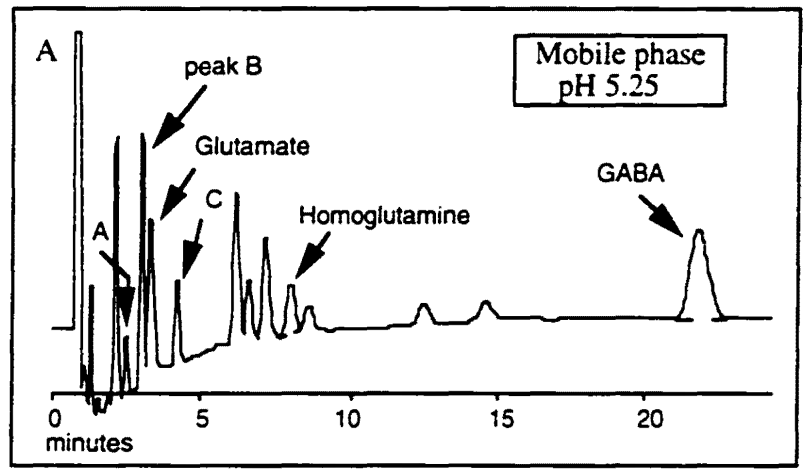


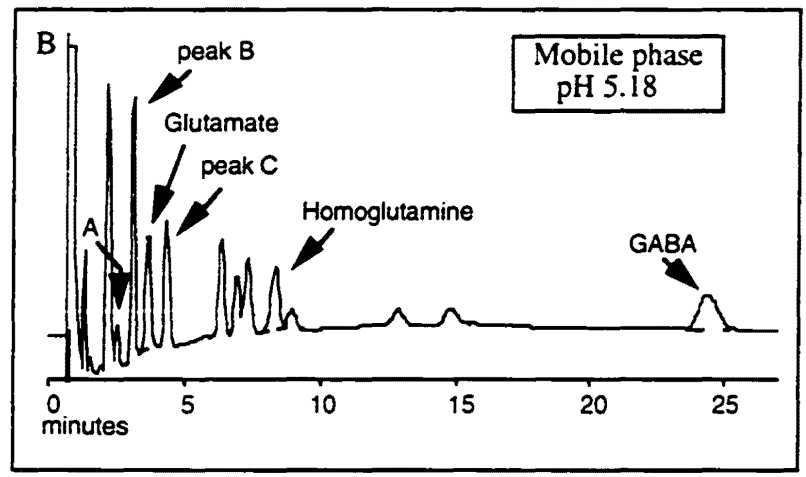
FIGURE 9 - The influence of the derivatizing sulphite concentration on the linearity of the standard curves of Glu and GABA relative to 10 pmol HGLN internal standard. Glu and GABA (1 to 50 pmol) were derivatized together with 10 pmol HGLN using OPA-SO_3 solutions containing two different concentrations of Na_2SO_3. A: Standards derivatized with an OPA solution containing 50 mM Na_2SO_3 generated a non-linear curve for Glu at Glu amounts above 25 pmol. B: Standards derivatized with an OPA solution containing 1.56 mM Na_2SO_3 enabled a near linear standard curve for Glu. Curves were assayed in triplicate (n=3) and error bars (may not be visible) represent SEM.

3. Effect of mobile phase pH on amino acid separation and elution

LCED analysis of tissue release samples generated many extraneous peaks which interfered especially with Glu separation. Many HPLC parameters contribute to the separation of amino acid derivatives on reverse-phase columns: the length of the column and its particle pore size, the organic modifier in the mobile phase, and the pH of the mobile phase. I found that Glu and GABA were very sensitive to eluant pH changes while early eluting extraneous material which compromised the quantitation of Glu was not. Pearson et al (1991) employed a mobile phase pH of 5.60, however, the Glu reaction product is better separated from interfering peaks at lower pH's. In figure 10 (A and B), chromatograms show samples of Glu and GABA releasate from rat CP tissue slices after stimulation by 50 mM KCl. Samples were derivatized in OPA solution containing 1.56 mM sulphite. Derivatized products in the tissue releasate yielded early eluting peaks in the chromatographic analysis labeled peaks A, B and C. Depending on the eluant pH, Glu could be made to co-elute with any of the peaks. Glu co-eluted with peak B under a mobile phase pH of 5.25 (Figure 10A). Reducing the mobile phase pH to 5.18, resulted in Glu eluting between peaks B and C, and separate from any interfering peaks (figure 10B).

Figure 11 shows the effect of several eluant pH's on the elution times of derivatized Glu, HGLN, GABA and extraneous material in the tissue releasate. Decreasing the pH from 5.54 to 5.18 increased the elution times of both Glu and GABA without greatly altering the elution times of any of the other column eluates. In figure 11A, Glu is shown co-eluting with adjacent peaks A and B at eluant pH's of 5.54 to 5.25, but at pH 5.18 Glu eluted between peaks B and C, separate from any co-eluting electroactive products. Figure 11B shows that the elution time of GABA increased as the mobile phase pH decreased, however the elution of HGLN was not altered. A more acidic mobile phase was not tested since this would further delay the elution of GABA. Because the optimal separation of the Glu reaction product from extraneous tissue constituents was observed at pH 5.18, this eluant pH was used routinely for estimation of Glu in the tissue extracts.





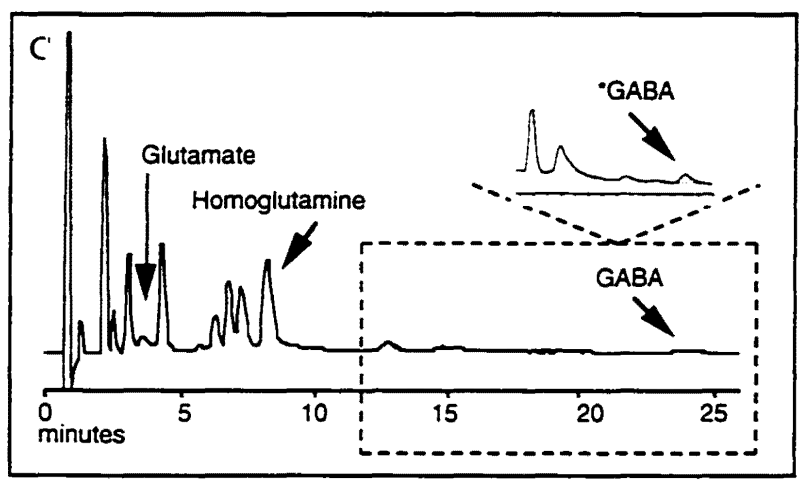
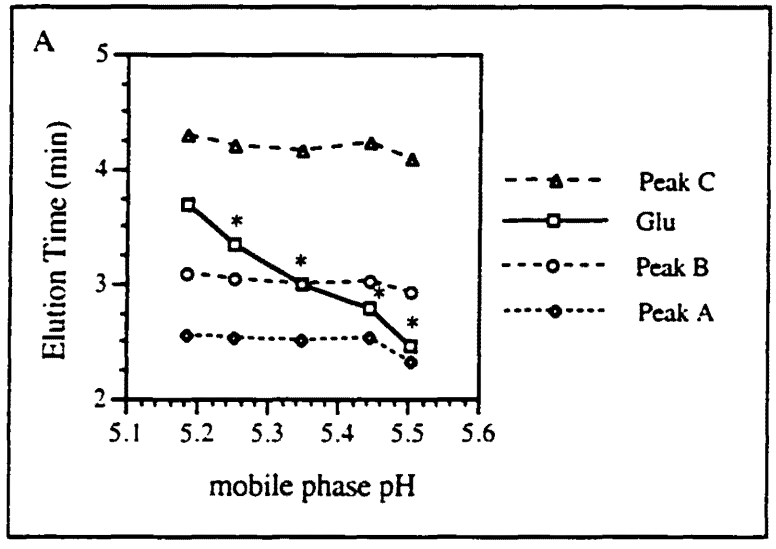


Figure 10. Chromatograms show tissue releasate of rat CP tissue slices under 50 mM KCl stimulation and under basal conditions. The releasate was derivatized for LCED analysis as described in the text. Extraneous material in the releasate sample yielded peaks (labeled A, B and C) on the chromatogram which interfered with the early detection of the derivatized Glu product. A: Under a mobile phase pH of 5.25, Glu co-eluted with peak B. B: The eluant pH was reduced to 5.18, and Glu eluted later, between peaks B and C. GABA elution time was also increased while all other peaks were not greatly affected. C: Typical chromatogram showing basal release of Glu and GABA as described in the text. * The inset shows the basal chromatogram from 12 minutes forward with the display sensitivity increased to better show the GABA peak. The eluant flow rate was 1.2 ml/minute.



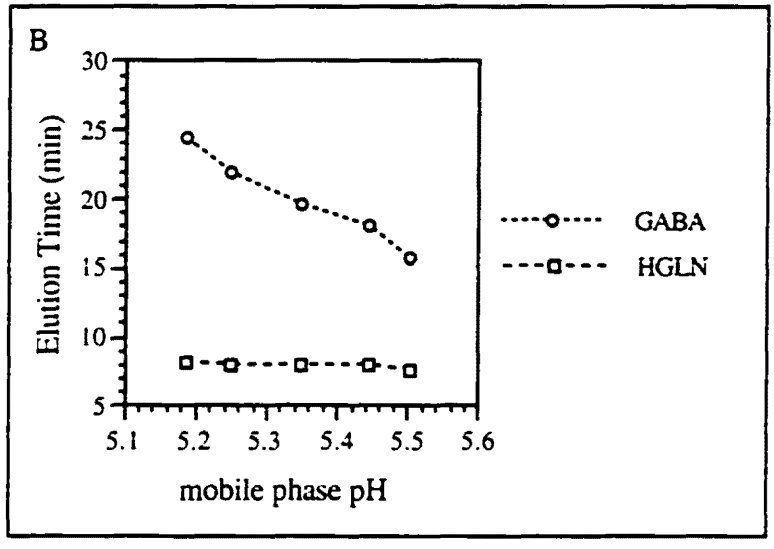


FIGURE 11 - The variation of elution times of LCED analyzed Glu and GABA derivatives from tissue releasate under various eluant pH's. A: The elution of derivatized Glu was shown to increase significantly with a consistent reduction in the eluant pH, while surrounding extraneous peaks A, B and C were not affected. This allowed Glu to elute in an ideal position, between peaks B and C. B: The elution time of derivatized GABA increased proportionally to the decrease in the mobile phase pH, while the elution time of the derivatized HGLN was not altered. Curves were assayed in triplicate (n=3, except at pH 5.18 n=5) and error bars (may not be visible) represent SEM. * Glu co-eluted with the adjacent peak.

B. Characterization of the sources of released glutamate and GABA from rat caudate-putamen tissue slices

Significant levels of Glu and/or GABA were released from the tissue slices by elevated levels of potassium, the potassium channel blocker 4-AP, and the Glu receptor agonist NMDA; exocytotic release was determined by showing that the stimulated release was dependent on the presence of calcium. Specific Glu and GABA reuptake inhibitors were used to evaluate the importance that amino acid reuptake transporters have on the tissue slice release assay design, and Ca^{2+} channels were selectively blocked with specific L-, N- and P-type channel blockers to determine which, if any, type of Ca^{2+} channel is specifically involved in the release of Glu and GABA from the CP tissue slices.

1. Depolarizing stimuli in the release of glutamate and GABA

The effect of increasing potassium concentration on the release of Glu and GABA from CP tissue slices is shown in figure 12A. 1 to 2 mg of tissue were incubated in 250 µl MKB for three separate incubations at 37° Celsius for 5 minutes. The final incubation was in MKB with varying concentrations of KCl from 5 to 100 mM. Release of both amino acids was dependent on the concentration of potassium between 20 and 100 mM. At KCl concentrations above 50 mM, more Glu than GABA was shown to be released, while GABA release increased more gradually from 50 to 100 mM KCl. Figure 12B shows the amounts of amino acids released by potassium as a percentage of maximum release from 100 mM KCl. The EC50 of KCl for the stimulation of Glu release was approximately 55 ±3 mM KCl, while the EC50 for GABA release was 51 ±3 mM KCl, based on the linear fits of individual curves. The curves indicate that the stimulated release of both Glu and GABA by 50 mM KCl was fairly equal in sensitivity to potassium depolarization. Experiments conducted over several months showed basal levels of Glu and GABA release to average approximately 325 ±35 and 144 ±10 pmol/mg protein, respectively, while potassium stimulated release (50 mM KCl) averaged approximately 4.6 ±0.2 and 5.5 ±0.3 nmol/mg protein for Glu and GABA, respectively. Potassium stimulated releasate samples

spiked with Glu and GABA standards gave recoveries of 98 ±1% (SEM; n=8) for Glu and 100 ±3% (n=8) for GABA.

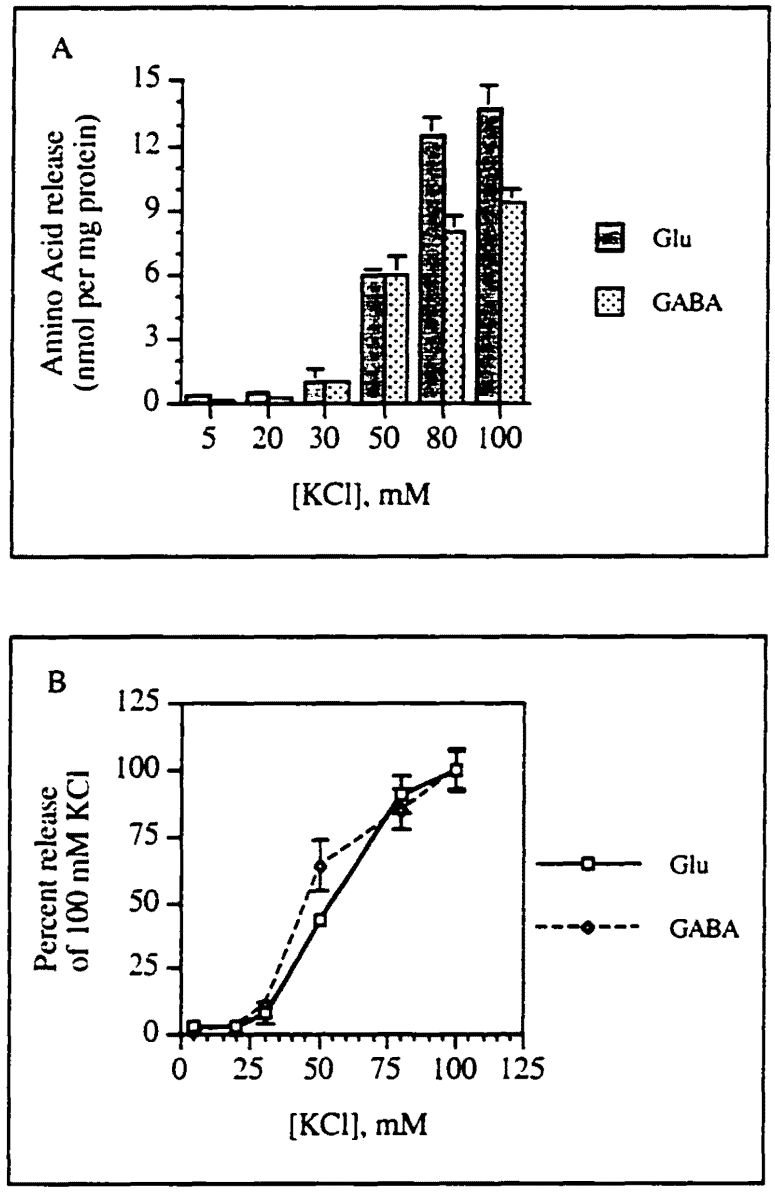


FIGURE 12 - Potassium stimulated release of Glu and GABA increased proportionally with the concentration of KCl. A: Basal and slightly above basal levels of amino acids were seen under 5 to 30 mM KCl, while a significant stimulation in release occurred with KCl concentrations above 50 mM. B: Percent potassium stimulated release of Glu and GABA as relative to 100% release from 100 mM KCl is shown. EC50 values of KCl stimulation of amino acid release were approximated from the linear portions of each curve. KCl had an EC50 of 55 ±3 mM for Glu release and 51 ±3 mM for GABA release. Samples were assayed in triplicate and error bars indicate SEM of three experiments (n=3).

The calcium-dependency of the release process activated by 50 mM potassium was studied. Tissue washes prior to the release assay were conducted with half the tissue (separated by volume of suspension) in MKB which contained no $CaCl_2$ and the other half which was in MKB. The control sample incubations were in MKB containing normal amounts of $CaCl_2$, and the tested sample incubations were in MKB containing 1 mM

EGTA with $CaCl_2$ removed. Glu release was reduced to 31 ±0.3% of control and GABA release was reduced to 17 ±1.2% of control release when calcium was removed from the medium. An influx of calcium into the neuron is believed to be essential for a depolarizing stimulus to elicit transmitter release (Kandel, 1991), and calcium dependency suggests that the majority of the amino acid release was by an exocytotic process.

Several other transmitter releasing mechanisms were evaluated in order to obtain more selective depolarizing agents other than potassium. The potassium channel blocker 4-AP, glutamate receptor agonists NMDA and AMPA, and the nicotinic-acetylcholine receptor agonist, epibatidine, were assayed. Both 4-AP (0.3 and 1.0 mM) and NMDA (10, 25,50 and 100 μM) stimulated amino acid release from the CP tissue slices; however, AMPA (10, 100 and 500 μM) and epibatidine (5, 50 and 500 nM) failed to yield significant stimulatory effects on the release of Glu or GABA. As indicated below in figure 13A, 4-AP released both amino acids above basal levels in a concentration dependent manner, yet was much more potent and selective at releasing GABA. Basal release of GABA increased nearly five-fold (488 ±51% control release) with 1.0 mM 4-AP, and was considerably higher than Glu release at all concentrations of 4-AP tested. Glu release was increased to 198 ±19% control release by the highest amount of 4-AP. NMDA also showed concentration dependent effects on stimulating the amino acid release; however, as shown in figure 13B, efficacy was much greater on Glu release. The maximum stimulatory effect on release was 280 ±39% control basal Glu release by 3.0 mM NMDA.

Ca^{2+} dependency studies were conduced on both NMDA and 4-AP stimulated release of Glu and GABA to determine if the release, as seen in figure 13, was occurring through exocytosis and neuron depolarization. The experiments were conducted as described previously; $CaCl_2$ was removed from the incubation media and 1 mM EGTA was added. Release of GABA by 0.3 and 1.0 mM 4-AP was reduced to 43 ±2% and 44 ±9% of control stimulated release, respectively (figure 14), indicating that at majority of the release was Ca^{2+} dependent. Selective stimulation of Glu release by NMDA was not dependent on

the presence of Ca^{2+}; release actually increased 7-fold under 1.0 mM and 5.5-fold with 3.0 mM NMDA when $CaCl_2$ was removed from the incubation media. NMDA stimulated release of Glu was also not blocked by Mg^{2+}. The lack of both Ca^{2+} dependency and Mg^{2+} sensitivity shows that the release of Glu was most likely not by an exocytotic process, nor mediated through activation of NMDA receptors. Other possible mechanisms of release are considered in the *Discussion* section.

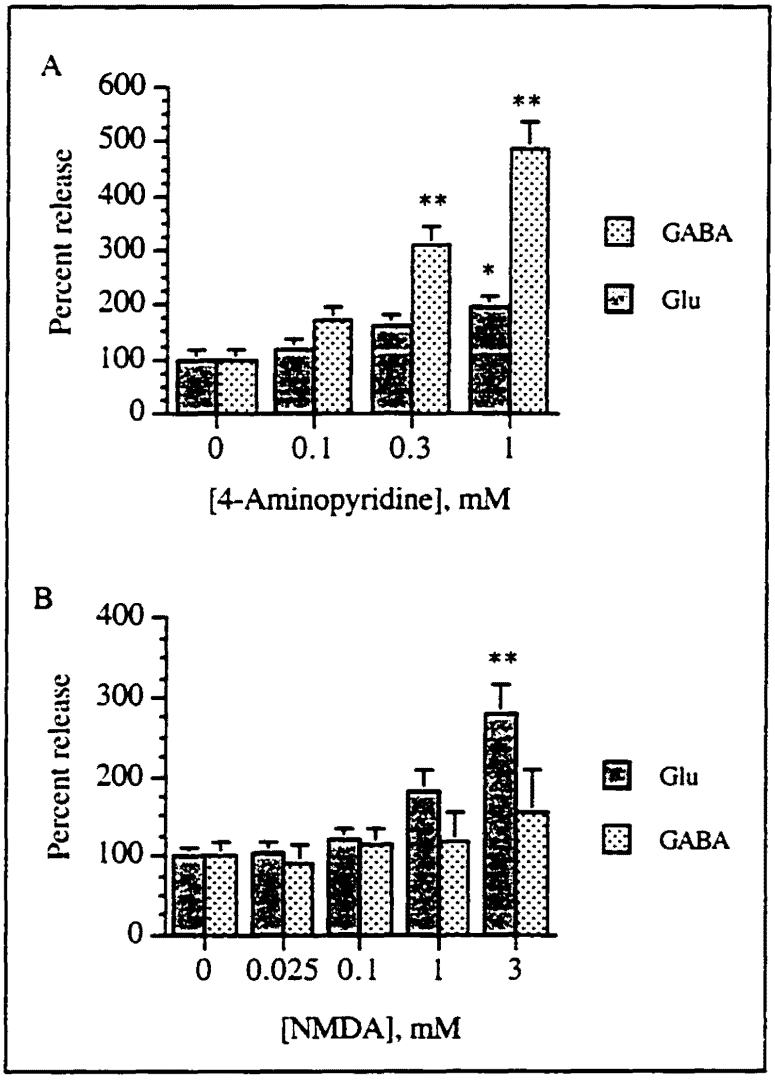


FIGURE 13 - 4-AP and NMDA stimulated release of amino acids from rat CP tissue slices under basal conditions is selective for GABA and Glu release, respectively. A: Basal GABA release was increased to a maximum of nearly 5-fold in a concentration dependent manner by 4-AP, while a much smaller effect was seen on Glu release. B: NMDA stimulated Glu release in a concentration dependent manner without greatly affecting basal release of GABA . A maximum effect was seen at 3 mM NMDA in which Glu release was about 280% of control. Samples of independent experiments were assayed in triplicate and graphs represent data using the following cumulative experiments (n): 4-AP in mM, 0 (13), 0.1 (3), 0.3 (13), 1.0 (12); NMDA in mM, 0 (5), 0.025 (3), 0.1 (3), 1.0 (4), 3.0 (5). ** $P<0.001$, * $P<0.01$ (ANOVA, Scheffe post hoc).

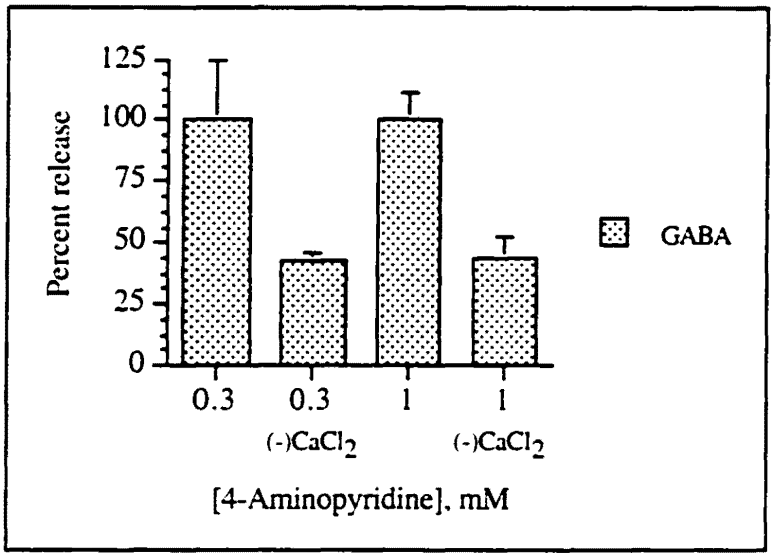


Figure 14 - Ca^{2+}-dependency of 4-AP stimulated release of GABA from rat CP tissue slices. Release of GABA by the two highest concentrations of 4-AP tested was reduced by over 50% when $CaCl_2$ was removed from the incubation media. This suggested that a majority of the release stimulated by potassium channel blockade was through an exocytotic process. Samples were assayed in triplicate, and error bars represent SEM of 3 independent experiments.

2. Effects of specific reuptake inhibitors on glutamate and GABA release

Experiments were conducted to test the effects of specific Glu and GABA reuptake inhibitors on release and to evaluate the importance that the amino acid reuptake transporters have on the release assay design. The GABA transporter blocker nipecotic acid and the Glu transporter blocker L-trans-2,4-PDC, among the most selective and potent reuptake inhibitors commercially available, were used separately in the following tissue slice release experiments. Their activity on both basal and stimulated release were measured using concentrations that are believed not to possess receptor agonist activity. Uptake inhibitors were added to the final two incubations, and release was measured from the final (third) incubation as described in the methods. As depicted in figure 15, nipecotic acid caused over a two-fold increase in the 50 mM KCl stimulated release of GABA and had no effect on the release of Glu, while the Glu reuptake inhibitor increased the stimulated release of Glu by about 50% and only slightly increased stimulated GABA release by 18 ±9% with 10 μM L-trans-2,4-PDC (figure 16). Because reuptake inhibitors have a tendency to act as substrates for the transporter and displace the transmitter from internal storage sites, their effects on basal amino acid release was tested, and this is shown in figure 17.

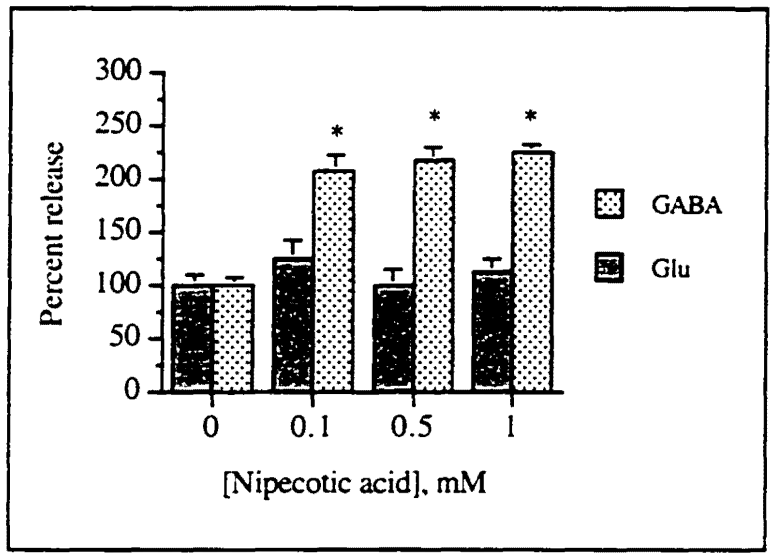


FIGURE 15 - Effect of nipecotic acid on stimulated release (50 mM KCl) of Glu and GABA. GABA release in the presence of nipecotic acid was over 200% of control release at all concentrations tested. Graph represents samples assayed in triplicate in 2 independent experiments with error bars indicating SEM.
* $P<0.05$ (ANOVA, Scheffe post hoc).

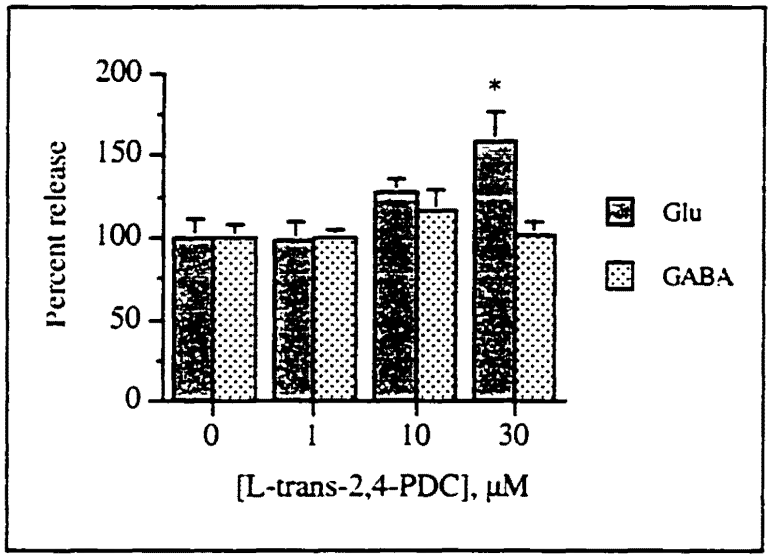


FIGURE 16 - Effect of L-trans-2,4-PDC on stimulated release (50 mM KCl) of Glu and GABA. 30 µM L-trans-2,4-PDC specifically increased the stimulated release of Glu by about 50%. Graph represents samples assayed in triplicate in 2 independent experiments with error bars indicating SEM.
* $P<0.05$ (ANOVA, Scheffe post hoc).

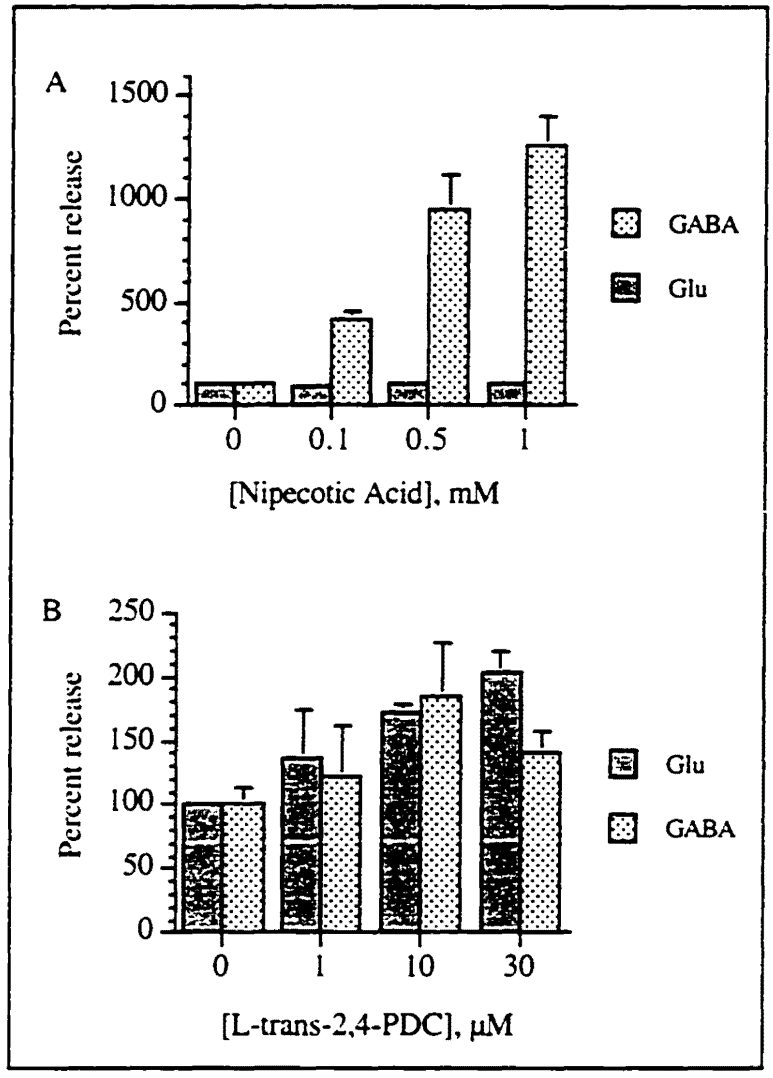


FIGURE 17- Effect of nipecotic acid and L-trans-2,4-PDC on basal release (5 mM KCl) of GABA and Glu. A: Basal release of GABA was increased 4 to over 12-fold by nipecotic acid in a concentration dependent manner. Basal release of Glu was not affected. B: L-trans-2,4-PDC increased basal Glu release to approximately 200% of control, while GABA release was increased slightly. Samples were assayed in triplicate with error bars indicating SEM.

The calcium dependency of nipecotic acid was also assayed to determine if the observed effects were mediated through uptake inhibition or by transmitter displacement. Figure 18, on the following page, shows the effects of nipecotic acid on basal and stimulated release of GABA with $CaCl_2$ removed from the media and 1 mM EGTA added. The 9-fold increase in basal GABA release by 0.5 mM nipecotic acid was shown not to be dependent on the presence of calcium; basal GABA release elevated by the uptake inhibitor actually increased by a third when calcium was removed (figure 18A). Figure 18B shows that the increase in potassium stimulated release of GABA by nipecotic acid was reduced when $CaCl_2$ was removed from the media. This apparent reduction in the uptake

inhibitor's effect was most likely a result of the potassium stimulation being dependent on calcium. As previously stated, potassium stimulated release of GABA was normally reduced to 17% of control release in the absence of Ca^{2+}, but in the presence of nipecotic acid release was reduced to only 65% of control.

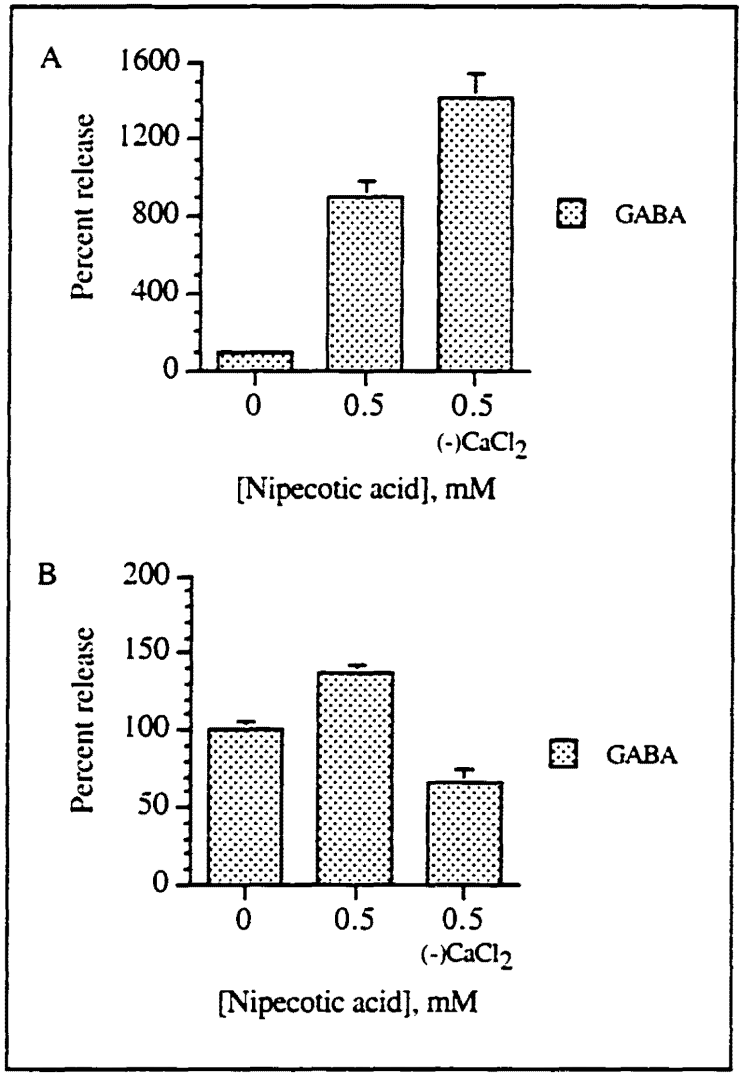


FIGURE 18 - Effect of nipecotic acid and its calcium dependency on basal and stimulated release of GABA. A: The effect of 0.5 mM nipecotic acid on basal GABA release was shown not to be dependent on the presence of calcium. Nipecotic acid's effect increased by a third when calcium was removed and 1 mM EGTA was added to the media. B: Nipecotic acid increased the potassium (50 mM) stimulated release of GABA to 150% control release, however when calcium was removed, release was reduced to 65% control release. The reduction in nipecotic acid's effect was most likely a result of the potassium stimulation being dependent on calcium and not nipecotic acid's effect being calcium dependent. See text for details. Nipecotic acid had no effect on Glu release in either case. Samples were assayed in triplicate with error bars indicating SEM.

3. Effects of specific Ca^{2+} channel blockers on glutamate and GABA release

Selective Ca^{2+} channels involved in the Ca^{2+}-dependent release of Glu and GABA from the tissue slices by 50 mM KCl and 4-AP were studied using specific Ca^{2+} channel antagonists: the L-, N- and P- type channel blockers nifedipine, CnTx GVIA and AgTx IVa, respectively. Channel blockers were added to the tissue during the second incubation in MKB and the third and final incubation containing the release stimulus. Results are shown in figure 19 and table 1 on the following pages. AgTx IVa yielded the greatest inhibitory effect on potassium stimulated release of both amino acids and GABA release by 4-AP. As shown in figure 19 and indicated in table 1, Glu and GABA release by 50 mM KCl was reduced to 33 ±5% and 18±0% control release, respectively, by 1 μM AgTx IVa. GABA release by both 0.3 and 1.0 mM 4-AP was reduced to 51 ±6% and 40±5%, respectively. Effect of AgTx IVa on 4-AP stimulated release of Glu is not shown because of the selectivity of 4-AP on GABA release (see figure 13). Basal release of Glu and GABA was not significantly affected by AgTx IVa. Table 1 shows that neither nifedipine nor CnTx GVIA caused significant inhibitory effects on Glu or GABA release from either stimulus. Values for AgTx IVa, taken from figure 19, are shown in table 1 for comparison purposes and suggests that Ca^{2+}-dependent release of Glu and GABA by potassium stimulation and GABA release by 4-AP stimulation was most likely mediated through P-type Ca^{2+} channels. These inhibitory effects of the P-type channel blocker yielded very similar results as initial Ca^{2+}-dependency studies when $CaCl_2$ was removed from the media and 1 mM EGTA was added (see figure 14 and its preceding text).

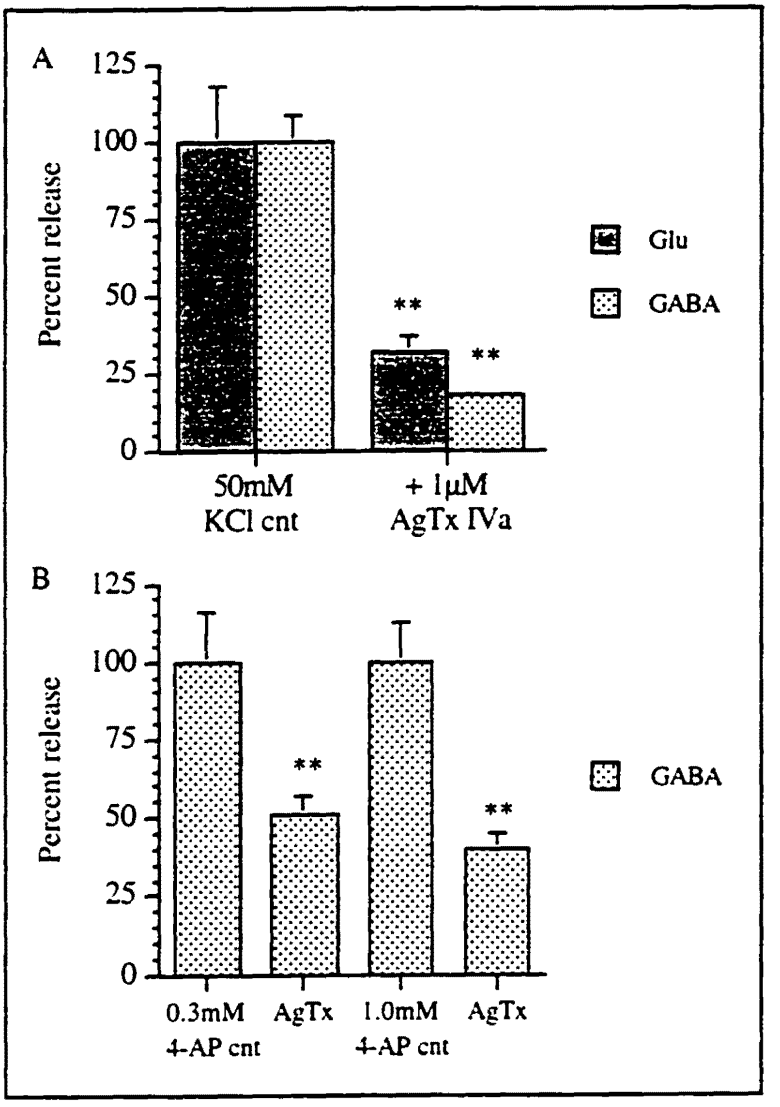


FIGURE 19 - Effect of 1 μM AgTx IVa on potassium stimulated release of Glu and GABA and 4-AP stimulated release of GABA from rat CP tissue slices. A: Glu and GABA release under 50 mM KCl stimulation was reduced to 33% and 18% control release, respectively (n=3). B: GABA release by both 0.3 mM and 1.0 mM 4-AP was reduced to 51% and 40% control release, respectively, by the P-type Ca^{2+} channel blocker (n=2). ** $P<0.005$ (ANOVA, Scheffe post hoc).

Stimulus	50 mM KCl		0.3 mM 4-AP	1.0 mM 4-AP	
% Cnt Release	Glu	GABA	GABA	GABA	n
3 μM Nifedipine	87 ±5	97 ±10	79 ±16	156 ±58	3
10 μM Nifedipine	72 ±13	141 ±8	93 ±0	97 ±16	2
100 μM Nifedipine	79 ±26	97 ±18	97 ±24	102 ±28	2
1 μM CnTx GVIA	106 ±33	101 ±19	222 ±89	85 ±2	2
1 μM AgTx IVa	33 ±5 **	18 ±0 **	51 ±6 **	40 ±5 **	3/2

TABLE 1 - Effects of specific Ca^{2+} channel blockers on potassium- and 4-AP- stimulated release of Glu and GABA from rat CP tissue slices. Both L- and N- type Ca^{2+} channel blockers nifedipine and CnTx GVIA, respectively, caused no significant reduction in the release Glu or GABA. Release in the presence of AgTx IVa was significantly reduced, suggesting that the P-type channel is the primary Ca^{2+} channel responsible for the Ca^{2+}-dependent release of the amino acids. Values are percent control release and represent samples assayed in triplicate in "n" separate experiments with "±" indicating SEM.
** $P < 0.005$ (ANOVA, Scheffe post hoc).

C. Local transmitter influence on the release of glutamate and GABA from rat caudate-putamen tissue slices

Potassium depolarization of neurons in CP tissue is not a specific stimulus of Glu and GABA release; other transmitters such as ACh and DA are most likely released under elevated potassium. A series of experiments were conducted using selective antagonists of striatal receptors to determine if local transmitters released upon potassium stimulation affected the measured release of Glu and GABA. Antagonists were added to block the receptors of specific striatal transmitters that may have been released along with the detected Glu and GABA, thereby reducing the influence of local transmitters on the release of the amino acids. The experimental design of these ligand studies was discussed in the *Methods* section.

Antagonists selective for Glu, GABA, ACh, DA, and the endogenous opiate receptors were used because these transmitters are known to have significant roles in striatal function, although local regulation of striatal Glu and GABA is not limited to these transmitters. The muscarinic-ACh, GABA and DA receptor antagonists showed significant effects on the release of the amino acids. All caused a decrease in release, but were selective for either Glu or GABA or specific subtypes of receptors. Atropine and saclofen yielded the greatest effects on release and their results are shown in figure 20 on the following page. Atropine, the muscarinic-ACh receptor antagonist, specifically inhibited Glu release in a concentration-dependent manner while not significantly altering GABA release; 10 μM and 100 μM atropine reduced the potassium stimulated release of Glu to 72 ±7% and 57 ±5% control release, respectively. The $GABA_B$ receptor antagonist, saclofen, significantly reduced both Glu and GABA release (figure 20B); the greatest concentration-dependent effects were seen with Glu. Glu release was reduced to 57 ±9% and 47 ±7% in the presence of 10 μM and 100 μM saclofen, respectively. GABA release was reduced to about 78 ±5% control release by both concentrations of saclofen.

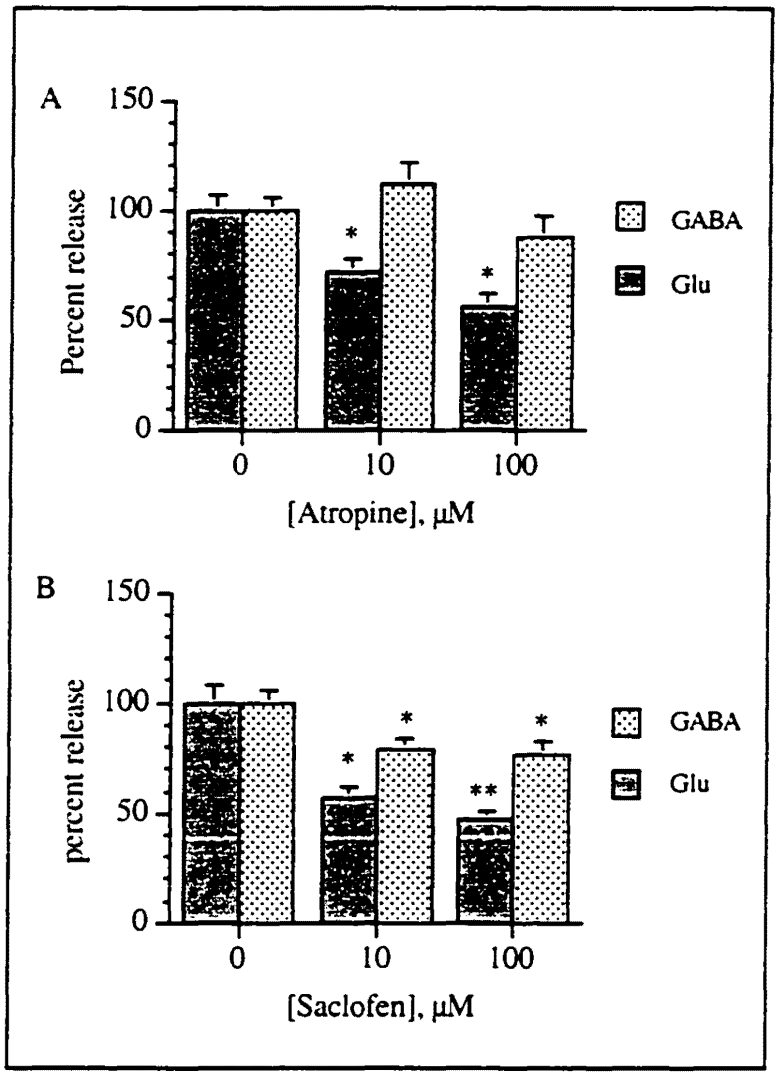


FIGURE 20 - Effects of atropine and saclofen on potassium-stimulated release of Glu and GABA from rat CP tissue slices. Antagonists were incubated with tissue slices in medium containing 50 mM KCl. A: Atropine, an antagonist at the muscarinic-ACh receptor, specifically reduced the release of Glu by nearly 50% of control without significantly affecting GABA release. B: The $GABA_B$ receptor antagonist, saclofen, caused significant reductions in both Glu and GABA release at concentrations tested. Graphs represent samples assayed in triplicate in 3 independent experiments with error bars indicating SEM. ** $P<0.005$, * $P<0.05$ (ANOVA, Scheffe post hoc).

Table 2 on the following page shows results from additional antagonist studies conducted in which results are presented as percent control release. The greatest effects on the amino acid release were seen with atropine and antagonists of both $GABA_A$ and $GABA_B$ receptors, although significance was only found with Glu release with the $GABA_A$ receptor antagonist bicuculline. The D_1 DA receptor antagonist, SCH 23390, reduced Glu release by about 25%, while the D_2 receptor antagonist, eticlopride, did not significantly affect the release of either Glu or GABA in these experiments. CTOP, the μ-opiate receptor antagonist, significantly reduced GABA release, but only at the lower

concentration, negating any concentration-dependent effect. BNTX, a δ_1-opiate antagonist, and naloxone, the non-selective opiate antagonist were assayed once as a screen for any other opiate mediate activity (data not shown), and they failed to significantly alter the release of Glu or GABA. None of the opiate receptor antagonists tested caused an increase in release, which indicated that no inherent endogenous opiate inhibitory activity influenced the measured release of Glu or GABA.

ANTAGONIST	RECEPTOR	Conc.	Glu	GABA	n
atropine	ACh muscarinic	10 μM	72 ±6 *	112 ±9	3
		100 μM	57 ±6 *	87 ±10	3
saclofen	$GABA_B$	10 μM	57 ±6 *	79 ±4 *	3
		100 μM	47 ±6 **	77 ±5 *	3
bicuculline	$GABA_A$	10 μM	60 ±6 *	98 ±9	2
		100 μM	58 ±3 *	83 ±4	2
CPP	NMDA	1 μM	74 ±10	88 ±6	3
		10 μM	91 ±9	85 ±6	3
CNQX	AMPA	1 μM	85 ±7	107 ±6	3
		10 μM	77 ±7	87 ±7	3
SCH 23390	D_1 DA	1 nM	71 ±6 *	92 ±7	3
		10 nM	74 ±8 *	104 ±16	3
eticlopride	D_2 DA	1 nM	69 ±10	77 ±7	3
		10 nM	71 ±10	105 ±20	3
CTOP	μ-opiate	100 nM	55 ±6	59 ±11 *	2
		1 μM	81 ±15	69 ±5	2

TABLE 2 - Receptor antagonists of local striatal transmitters: their effects on 50 mM KCl stimulated release of Glu and GABA from rat CP tissue slices. Glu and GABA, measured by HPLC analysis, were released from the tissue by potassium stimulation presumably with other transmitters that were sensitive to potassium-induced depolarization. The effects mediated by antagonists are indicated as percent release of control (50 mM KCl stimulation in the absence of antagonist compared to release in the presence of the antagonist). Results are from samples assayed in triplicate from "n" independent experiments with "±" indicating SEM. ** $P<0.005$ and * $P<0.05$ (ANOVA, Scheffe post hoc).

Several striatal receptor agonists were assayed for their ability to either inhibit the release of the amino acids from potassium stimulation or stimulate release of the amino acids above basal levels. Selective agonists of the three major classes of opiate receptors were assayed for their ability to inhibit release. The results of these experiments are shown in table 3 below. DAMGO, a μ-selective agonist, DPDPE, a δ_1-selective agonist, and the κ-selective agonist U69593, were assayed. Of the three agonists assayed, the selective δ-receptor ligand induced a 30-40% reduction of Glu release ($P<0.05$), although GABA release was not affected in a statistically significant manner. The δ_2-selective agonists, DSLET and deltorphin II, were assayed once (not shown), and they failed to yield any concentration-dependent reduction in Glu or GABA release that would have warranted further experiments. The μ-selective agonist, DAMGO (0.1 and 1 μM), and the κ-selective agonist U69593 (0.1 and 1 μM) did not significantly affect potassium stimulated Glu and GABA release.

AGONIST	RECEPTOR	Conc.	Glu	GABA	n
DAMGO	μ-opiate	0.1 μM	82 ±4	121 ±13	4
		1 μM	89 ±9	95 ±11	4
DPDPE	δ_1-opiate	0.1 μM	61 ± 6 *	98 ±11	2
		1 μM	72 ± 8 *	114 ±12	2
U69593	κ-opiate	0.1 μM	105 ±19	97 ±9	3
		1 μM	86 ±12	84 ±7	3

TABLE 3 - The effects of selective opiate receptor agonists on 50 mM KCl stimulated release of Glu and GABA from rat CP tissue slices as assayed by HPLC. Only DPDPE selectively reduced Glu release, while GABA release was not significantly altered by any of the agents. Results are from samples assayed in triplicate from "n" independent experiments with "±" indicating SEM. * $P<0.05$ (ANOVA, Scheffe post hoc).

The D_1 DA receptor agonist, R(+)-6-bromo-APB HBr (6-Br-APB), was assayed for its potential stimulatory effects on increasing the amino acids release above basal levels and above levels stimulated by elevated potassium. No significant increase in either Glu or

GABA was achieved by the agonist under basal or stimulatory conditions. The results are shown below in table 4.

Release conditions	[6-Br-APB], nM	Glu	GABA	n
5 mM KCl	20	151 ±15	176 ±75	3
	40	134 ±26	123 ±27	3
50 mM KCl	20	83 ±1	79 ±10	2
	40	80 ±5	83 ±5	2

TABLE 4 - Effects of D_1 DA receptor stimulation by 6-Bromo-APB on basal and stimulated release of Glu and GABA from rat CP tissue slices.

Quinelorane, a D_2 DA receptor agonist, was assayed for its potential inhibitory effects on stimulated release of the amino acids. Neither Glu nor GABA release evoked by 50 mM KCl was significantly affected by either 30 or 100 nM quinelorane, and GABA release induced by 0.3 and 1.0 mM 4-AP was also not significantly reduced by the D_2 DA agonist. Because atropine, the muscarinic-ACh receptor antagonist, showed significant inhibitory effects on potassium stimulated release of Glu, it was thought that activation of muscarinic receptors might release Glu above basal levels. Bethanechol, a muscarinic receptor agonist resistant to cholinesterase activity, at 1 mM had no effect on the basal release of either amino acid. The highest concentration tested, 5 mM, stimulated Glu release to 143 ±7% and GABA release to 139 ±14% control release, however this did not reach statistical significance.

D. Endogenous GABA release from fetal rat primary striatal cultures

Striatal neurons were cultured for 7 days prior to the GABA release experiments. Figure 21, on the following page, shows typical chromatograms of the LCED analysis of the derivatized culture releasate. Unlike releasate from the CP tissue slices in which both Glu and GABA were detected, only GABA was detected in the PSC releasate (figure 21A). Following the culture incubation in stimulus media, a fourth and final incubation was conducted in 0.2 N HCl. This "acid wash" incubation presumably released all contents of the neurons causing large amounts of GABA, as well as Glu, to be extracted into the wash. The large quantities of stored Glu and GABA made it necessary for the acid wash samples to be diluted 8-fold with water for accurate LCED analysis. Figure 21B shows a typical chromatogram of an acid wash sample in which both Glu and GABA were detected. Glu was most likely contained in the GABAergic neurons as a precursor of GABA and not as releasable neurotransmitter, hence its insensitivity to depolarizing stimuli. The measurement of GABA in the acid wash samples was necessary for data to be reported as fractional release. The internal standard HGLN was added to the samples prior to the sample derivatization.

1. Release of GABA by depolarizing stimuli

GABA release from the PSC was stimulated by potassium, 4-AP and Glu. Figure 22 shows typical dose response curves for GABA release by these three transmitter-releasing agents. As described in the methods, the cultured neurons underwent four incubations: the first two were in MKB, the third was in various concentrations of either KCl, 4-AP or Glu, and the fourth was in 0.2 N HCl. Basal release was measured from releasate of the second incubations. GABA release reached a maximum within all the concentration ranges tested for each stimulus. Potassium reached its maximum effect with 80 mM KCl (figure 22A), 4-AP with 0.3 mM (figure 22B) and Glu with 25 μM (figure 22C). Note that 25 to 100 μM Glu was as effective as KCl on stimulating GABA release (this was shown in several

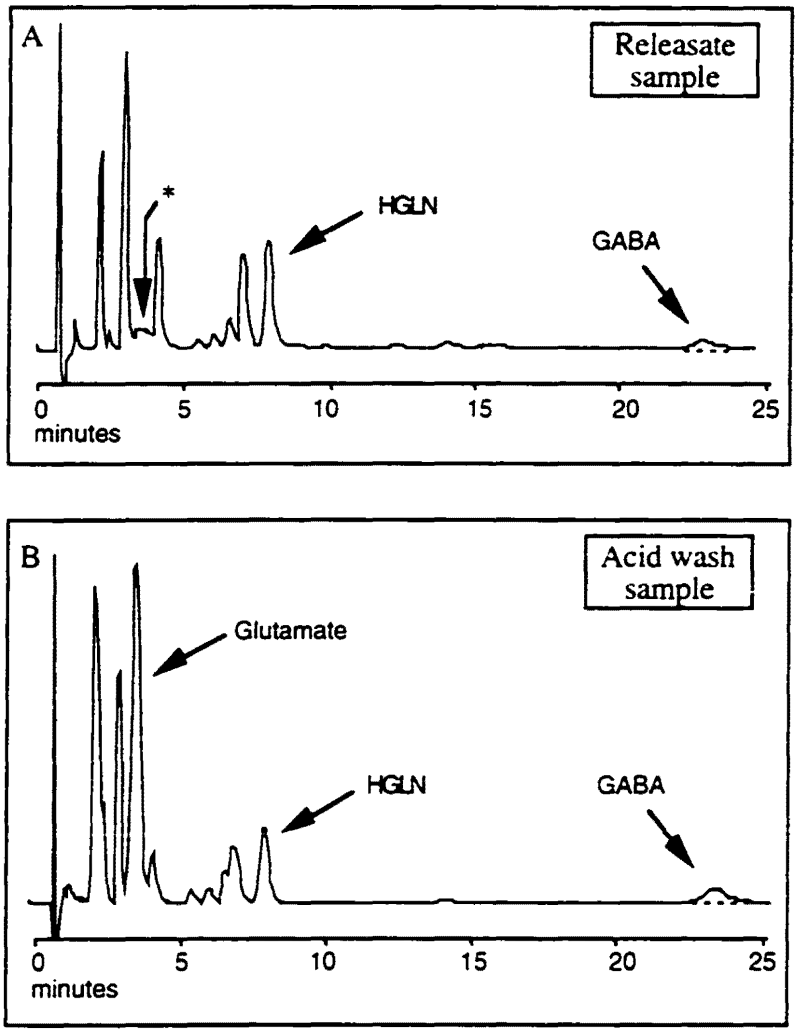


FIGURE 21 - Typical chromatograms of LCED analysis of the derivatized culture releasate. Samples were collected, derivatized and injected into the HPLC as described in the text. A: Only GABA, and not Glu, was detected in typical stimulated releasate samples; here, release was induced from 50 mM KCl stimulation. * indicates where a Glu peak would be seen if Glu were released into the sample media. B: Both Glu and GABA were detected in an analysis of a diluted acid wash sample. Dilution of the sample was required because large amounts of the amino acids were released into media following exposure of the PSC to 0.2 N HCl. In both chromatograms the internal standard, HGLN, was added to the samples prior to derivatization.

additional experiments as well). These curves were conducted once each to test for a concentration-dependent trend in the GABA releasing actions of the stimuli. The effects of both KCl (50 mM) and 4-AP were shown to be Ca^{2+}-dependent, however only the lowest concentration of Glu (10 μM) required Ca^{2+} to achieve its effect. Ca^{2+}-dependency was shown by conducting a typical culture release assay except that throughout the experiment $CaCl_2$ was removed from the MKB and 1 mM EGTA was present. GABA release by 50 mM KCl was reduced to 31 ±2% control release; release by 0.3 and 1.0 mM 4-AP was

reduced to 25 ±2% and 30 ±2% control release, respectively; and release by 10 μM Glu was reduced to 11 ±0% with $CaCl_2$ absent from the media.

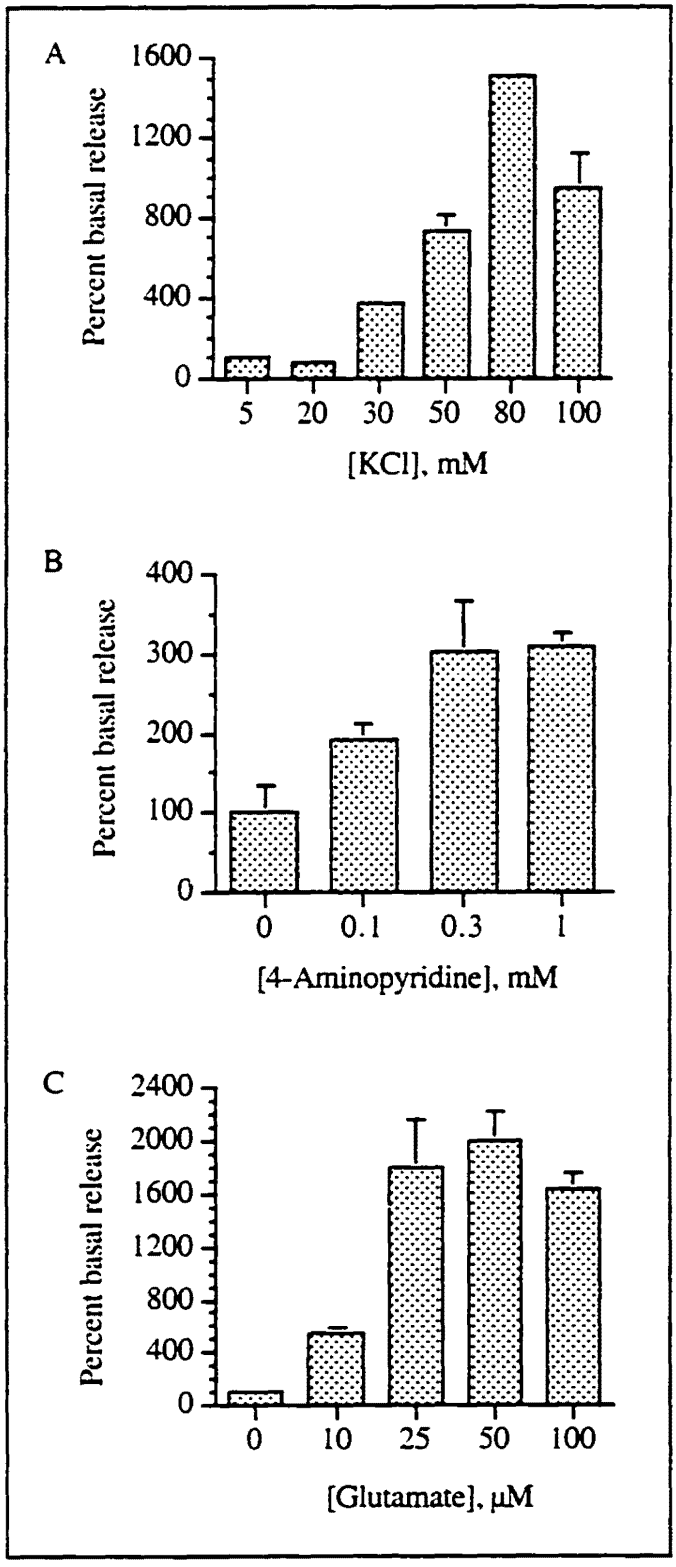


FIGURE 22 - Release of GABA from PSC by potassium, 4-AP and Glu as a percentage of basal release. A: Release stimulated by potassium was dependent on the concentration of KCl from 20 to 100 mM, and maximum release was achieved with 80 mM KCl. Release by 50 mM KCl was Ca^{2+}-dependent and used throughout further experiments when potassium stimulated release of GABA was assayed. B: 4-AP stimulated release of GABA in concentration ranges from 0.1 to 1.0 mM. The effects of both 0.3 and 1.0 mM 4-AP were Ca^{2+}-dependent. C: Glu was the most effective stimulator of GABA release and reached its maximum effect at 25 μM. However, release by 10 μM Glu, the lowest amount tested, was the only concentration requiring the presence Ca^{2+}. Samples were assayed in duplicate and error bars (may not be visible with 80 mM KCl) represent SEM.

2. Dopamine receptor activity on basal and stimulated release of GABA

The effects of stimulating D_1 and D_2 DA receptors on the release of GABA was studied using DA receptor agonists 6-Br-APB and quinelorane, respectively. 6-Br-APB was assayed for its stimulatory activity on basal release, potassium stimulated release and release stimulated by 4-AP. Figure 23, below, shows the agonist's effect on stimulating GABA release above basal levels. 6-Br-APB, at 3, 30 and 60 nM, was added to individual wells of the cultured neurons in the second incubation from which basal release was assayed. Of the three concentrations tested, the maximum effect was reach with 30 nM 6-Br-APB, and statistical significance was established through separate additional experiments ($P< 0.01$, n=7). Percent control basal release was 94 ±1% for 3 nM, 175 ±18% for 30 nM and 183 ±1% for 60 nM 6-Br-APB.

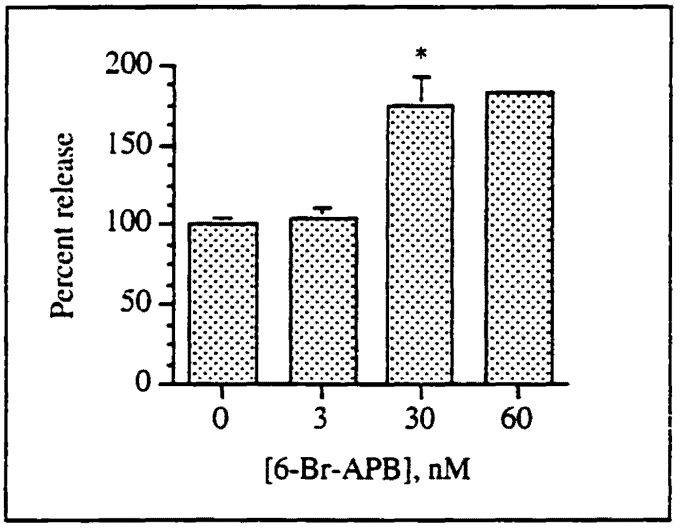


FIGURE 23 - Effect of D_1 DA receptor stimulation on basal release of GABA from fetal rat PSC. 6-Br-APB stimulated the release of GABA above basal levels by increasing release to approximately 180% control release at the two highest concentrations tested. Graph represents samples assayed in duplicate with error bars indicating SEM of duplicates or separate experiments (n): 3 nM (1), 30 nM (7) and 60 nM (1). * $P< 0.01$ (paired t test).

Specific receptor mediated activity was verified by assaying the effects of 6-Br-APB in the presence of a selective D_1 DA receptor antagonist, SCH 23390. Figure 24 on the following page shows that the release of GABA by 30 nM 6-Br-APB was significantly reduced to near control levels in the presence of 30 nM SCH 23390. The agonist

stimulated release to 175 ±18% control basal release, but in the presence of the antagonist, release was reduced to 91 ±8% control release. SCH 23390, alone, had no effect on the basal release of GABA. This verified that the significant stimulatory effect of basal GABA release by 6-Br-APB was receptor mediated through D_1 DA receptor stimulation.

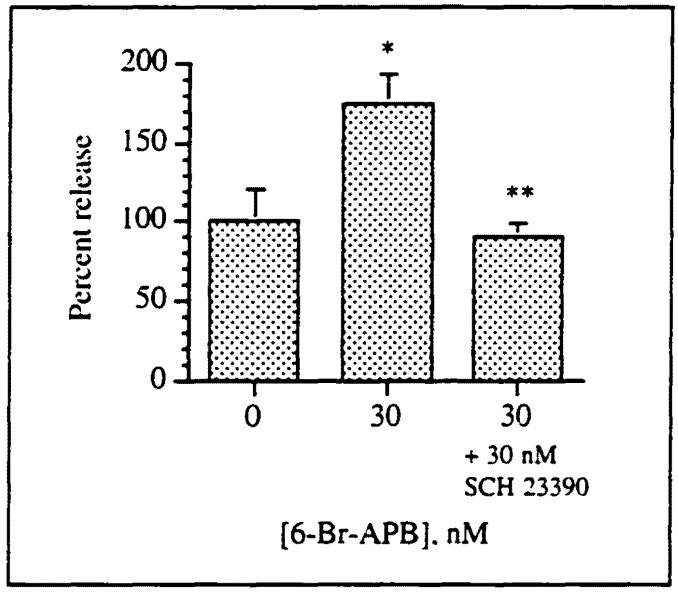


FIGURE 24 - SCH 23390 blocked the 6-Br-APB stimulation of GABA release from fetal rat PSC. The stimulated release of GABA above basal levels by D_1 DA receptor activation was significantly blocked and reduced to control levels by the D_1 DA receptor antagonist, SCH 23390. This confirmed that the stimulated release of GABA by 6-Br-APB was through D_1 DA receptor activation. Graph represents samples assayed in duplicate from independent experiments with error bars indicating SEM: 30 nM 6-Br-APB (n=7), + 30 nM SCH 23390 (n=2).* $P<0.01$, ** $P<0.005$ (paired t test).

The effect of 30 nM 6-Br-APB on potassium and 4-AP stimulated release of GABA was also assayed. The agonist increased 50 mM KCl stimulated release to 122 ±8% control release ($P<0.1$, n=5), but failed to mediate an effect on release stimulated by 0.3 mM 4-AP (107 ±17%, n=3). Since no statistical significance of $P<0.05$ was established in either case, agonist effects in the presence of the antagonist were not tested.

The inhibitory effects of D_2 DA receptor stimulation was tested against potassium stimulated release of GABA from the PSC. Quinelorane, at 30 and 100 nM, was incubated with individual wells of cultured neurons in the second and third incubation steps of the release experiment. Elevated potassium (30 and 50 mM KCl) was added to release GABA in the third incubation, and this releasate was assayed for its GABA content. In two separate experiments each, quinelorane did not significantly reduce the potassium stimulated release of GABA.

3. μ-Opiate receptor activity on stimulated release of GABA

The selective μ-opiate receptor agonist, DAMGO, was assayed for its ability to inhibit potassium and 4-AP stimulated release of GABA from the PSC. 1 μM DAMGO was added to the cultures in the second and third incubations of the release experiment; stimulus was applied to the cultures in the third incubation and its releasate was assayed for GABA content. GABA release induced by either 50 mM KCl or 0.3 mM 4-AP was not affected by μ-opiate receptor stimulation; potassium stimulated release in the presence of DAMGO was 105 ±2% control release and release stimulated by 4-AP was 110 ±10% control release. The opiate receptor antagonist, naloxone, was used in separate experiments to test for the release of endogenous opiates and their activity on the release of GABA from the cultures. 10 μM naloxone, present in the second and third incubations, failed to significantly increase 50 mM KCl stimulation of GABA release (119 ±12% control release, n=4) which indicated that under these experimental conditions, endogenous striatal opiates were not affecting GABA release.

GABA released into the culture media may have been acting on striatal GABA autoreceptors and thus reducing the amount of stimulated GABA release detected in the releasate. This reduction in release might have masked any inhibitory effect that μ-opiate receptor stimulation would have on stimulated GABA release. To test this hypothesis, I first examined the autoreceptor regulation of GABA release. $GABA_A$ and $GABA_B$ receptor antagonists were assayed for their effects on potassium stimulated release. An increase in the stimulated release above control levels in the presence of a specific antagonist would indicate that the released GABA is acting on striatal GABA receptors and inhibiting its own stimulated release. In figure 25 on the following page, the effects of selective GABA receptor antagonists on 50 mM KCl stimulation of GABA release are shown. Only the $GABA_A$ receptor antagonist, bicuculline, caused a significant increase in potassium stimulated release of GABA (143 ±18% control release). Release in the presence of the $GABA_B$ receptor antagonists, saclofen and 2-OH saclofen, was 108 ±9% and 129 ±22%

control release, respectively. Bicuculline and 2-OH saclofen together caused potassium stimulated release to increase 57 ±26%; however, the large variance in these samples prevented the treatment from reaching statistical significance ($P>0.05$).

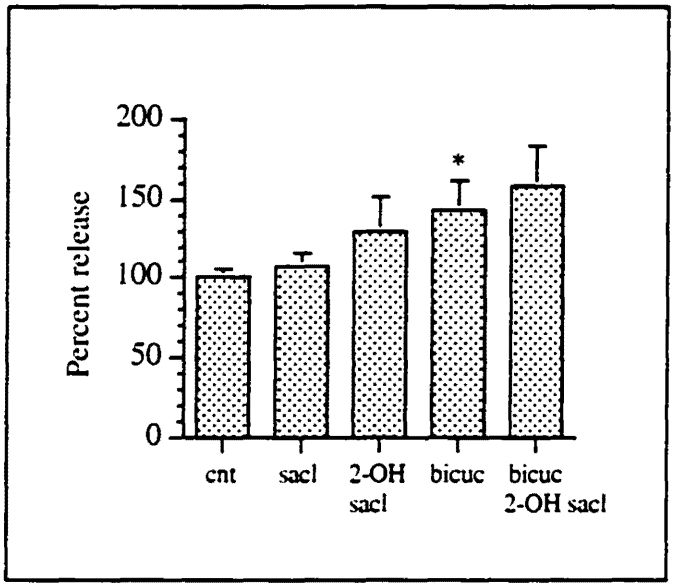


FIGURE 25 - The effect of GABA receptor antagonism on 50 mM KCl stimulated release of GABA from fetal rat PSC. 100 μM each of $GABA_B$ receptor antagonists, saclofen (sacl) and 2-OH saclofen (2-OH sacl), and the $GABA_A$ receptor antagonist bicuculline (bicuc) were assayed for their effects on potassium stimulated release of GABA. $GABA_B$ receptor antagonism caused no significant increases in GABA release, while the $GABA_A$ receptor antagonist increased the stimulated release of GABA by nearly 50%. Bicuc and 2-OH sacl together, caused no significant synergistic effect. Results are from independent experiments (n) with error bars indicating SEM: sacl (n=2), 2-OH sacl (n=4), bicuc (n=6), and bicuc/2-OH sacl (n=3). * $P<0.05$ (paired t test).

Inhibitory effects of μ-opiate receptor activation on the bicuculline increase in potassium stimulated release was tested (figure 26). Although DAMGO (1 μM) appeared to reduce GABA release in the presence of 100 μM bicuculline, the reduction was not statistically significant ($P>0.05$). Bicuculline increased the stimulated release to 143 ±18% control, and in the presence of DAMGO, bicuculline's effect was 107 ±15% control release.

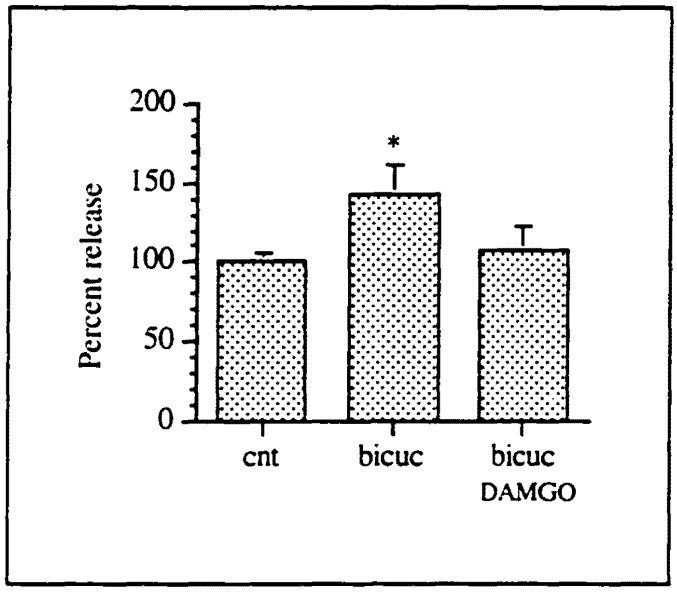


FIGURE 26 - Effect of DAMGO on the bicuculline increase in 50 mM KCl stimulation of GABA release from fetal rat PSC. The $GABA_A$ receptor antagonist, bicuculline (bicuc, 100 μM), increased potassium stimulated release of GABA to 143% of control release, however in the presence of 1 μM DAMGO, this effect was reduced to 107%. Statistical significance was not reached for the DAMGO effect, however. Results are from independent experiments (n) with error bars indicating SEM: bicuc (n=6) and bicuc/DAMGO (n=5).
* $P<0.05$ (paired t test).

DISCUSSION

A. HPLC and electrochemical detection of glutamate and GABA

The initial objective of this project was to obtain a reproducible assay that would allow endogenous Glu and GABA released from striatal tissue to be measured by sensitive and reliable means. Because measuring endogenous Glu and GABA simultaneously from one releasate sample is a fairly new concept and very important for the basis of this project, a majority of the time spent on preliminary experimentation went into the development of the assay parameters. The methods employed by Pearson et al (1991) and Smith and Sharp (1994a,b) were used in establishing the basic method for HPLC and electrochemical detection of amino acids, but the methods required modification to improve the detection reliability. More recent work by Rowley et al (1995) was used to verify these unique improvements for measurement of endogenous Glu and GABA through LCED analysis of their OPA-SO_3 derivatives. The revised method has recently been published (Phillips and Cox, 1997).

Electrochemical analysis of sulphites, originally described by Jacobs (1987), has been used frequently for amino acid detection in brain homogenates (Pearson et al., 1991) and microdialysis preparations (Smith and Sharp, 1994b and Rowley et al., 1995). Several problems with this method of HPLC analysis were apparent: previously used amino acid internal standards compromised the efficiency of the LCED analysis, and amino acids such as Glu that elute early in the chromatogram are susceptible to interference by excess sulphite as well as other reaction products and tissue constituents.

The internal standards, BABA (Pearson et al., 1991) and HSER (Donzanti and Yamamoto, 1988), were first evaluated because of their previous reported use. BABA yielded a weak detector response and increased the chromatographic analysis time, while HSER co-eluted with extraneous material in the standard injections under my HPLC parameters. It should also be noted that HSER is an amino acid intermediate found

endogenously in biochemical reactions (Voet and Voet, 1990); this may compromise its use as an internal standard in the LCED analysis of tissue amino acid levels. An internal standard is an amino acid that is combined with the sample containing Glu and GABA; it undergoes derivatization and is injected into the HPLC with each sample. It serves several important functions: it verifies that the sample separation and detection is running normally, that the derivatization reaction is occurring properly, and it serves to establish a standard curve for quantifying unknown amounts of Glu and GABA released from the tissue. The internal standard used in quantifying Glu and GABA release ideally should be a non-endogenous amino acid that elutes between Glu and GABA. HGLN is not an endogenous amino acid, and its structure indicated to me that its derivatized product would likely elute between Glu and GABA. HGLN was found to elute later than HSER and just after the extraneous column eluant that co-eluted with HSER. Stock solutions of HGLN tested repeatedly showed stable elution times and gave consistent detector responses over many experiments. It was subsequently used in all LCED analyses of Glu and GABA release from the tissue slices and GABA release from the PSC.

The amount of sulphite used in the derivatization media was shown to be critical in the baseline elution of Glu, and hence the standard curve of Glu. Rowley et al (1995) discussed sulphite contamination briefly, and reduced the amount of derivatizing agent only when dialysis samples were derivatized. I found it beneficial to specifically reduce the sulphite much further and maintain a consistent amount used in both the measurement of standards for standard curves and in the sample releasate analysis. The reduction in sulphite concentration did not alter the magnitude of the detector response to derivatized amino acids including Glu, GABA and HGLN. It appears the concentration is still in large excess for the derivatization reaction. I also used a dual analytical cell to apply two positive potentials to the column eluates: an initial low voltage was used to screen out potential interference, while the derivatized amino acids were detected through the second potential which was higher. The first applied potential of 350 mV was helpful in reducing the

detected response of the extraneous peak which eluted just prior to the HGLN peak (data not shown).

Adjustment of the sulphite concentration in the derivatization solution led to significant improvements in the amino acid standard curves, however releasate samples from both *in vitro* and *in vivo* amino acid analysis produced extraneous materials which were detected in each LCED analysis of OPA-SO_3 derivatized amino acids. Most of this material produced peaks seen very early in the chromatogram, around the elution time of the Glu reaction product. This had little bearing on GABA detection but significantly compromised the quantitation of Glu. I showed that Glu and GABA were sensitive to mobile phase pH changes while most other electroactive eluates were not, within the pH range tested. Reduction of the sulphite concentration used in the derivatization and appropriate adjustment of the mobile phase pH gave baseline separation of both Glu and GABA. This ultimately enabled a more accurate measurement of the derivatized Glu product and a more reliable standard curve for Glu.

The *in vitro* multi-well filter-plate release assay was significant in the study of Glu and GABA release primarily because it enabled the analysis of numerous samples from various pharmacological treatments within individual experiments. Other *in vitro* tissue slice release assays were considered. Superfusion, which is commonly used in radiolabeled transmitter release analysis (i.e. Jacocks and Cox, 1992), generates large amounts of separate releasate samples (superfusates), and transmitter release of individual samples (and the pharmacological effect) is studied over a series of superfusates. LCED analysis of endogenous amino acid release through superfusion would require a significant amount of time to analyze the separate superfusates of individual samples. A multi-well plate system for the study of Glu and GABA release from tissue slices was more applicable for this project, making it possible to obtain the required data within a reasonable period of time. Several trial experiments were conducted using an alternative multi-well plate assay. This assay, previously used by Werling et al (1988) to study [^{3}H]-dopamine release from rat

striatum and cortex tissue slices and Kim and Cox (1993) for the study of [^{3}H]-norepinephrine release from rat cortex tissue slices, employs a 24-well plate with a removable basket-insert tray to handle tissue slices. Larger wells are used in this system which require larger amounts of buffer, drug or peptide, and tissue, compared to the 96-well microtiter plate. Considering the size of the rat striatum and the study of endogenous transmitters, it was much more feasible to use the 96-well microtiter plate which has smaller wells for tissue and buffer distribution; this permits the recovery of a more "concentrated" releasate. The most notable disadvantage of studying transmitter release with this type of multi-well assay plate procedure, is that transmitters are released into a "static-well" environment. Thus Glu and GABA are released into the buffer containing the tissue for the duration of the tissue incubation or release period, unlike superfusate which is nearly instantaneously superfused from the tissue, depending on the flow rate of superfusion. A static-well releasate system may permit the released transmitters to augment or reduce their own release through autoreceptor regulation or presynaptic re-accumulation. The disadvantage of this approach is that the observed "release" is a sum of release and reuptake process. The advantage is that this may closely parallel the *in vivo* situation.

B. Release of glutamate and GABA from rat caudate-putamen tissue slices by depolarizing stimuli

Release of Glu and GABA was stimulated by elevated potassium, 4-AP and NMDA. Potassium stimulated release of both amino acids in a concentration dependent manner, while 4-AP selectively released GABA, and NMDA was more efficacious toward Glu release. Release stimulated by 50 mM KCl and 4-AP was Ca^{2+}-dependent, however NMDA stimulated release of Glu was not; NMDA evoked release of Glu was also not affected by the presence of Mg^{2+}.

The Ca^{2+}-dependency of Glu and GABA release suggests that a majority of release was occurring primarily through exocytosis (Kandel, 1991). However, 31% of Glu

release and 17% of GABA release evoked by potassium was not Ca^{2+}-dependent, as was about 44% of GABA release stimulated by 4-AP. The Ca^{2+}-independent release seen with these depolarizing agents may have resulted from a portion of release being dependent on Na^+. Norris et al (1983), Carvalho et al (1986), Bernath and Zigmond (1990) and others have shown that depolarization evoked transmitter release is blocked by Ca^{2+} channel antagonists even in the absence of external Ca^{2+}. Bernath (1992) interprets this as meaning ionic influxes still occur through Ca^{2+} channels when external Ca^{2+} is absent. He, as well as others, also suggest that transmitter release evoked through depolarization in the absence of Ca^{2+} is solely dependent on the presence of external Na^+, and that Na^+ is transported through voltage-dependent Ca^{2+} channels during depolarization, particularly when residual Ca^{2+} is bound by chelators such as EGTA (see for review Bernath, 1992). The Na^+-dependency of release in the absence of Ca^{2+} was not evaluated in my experiments. However, it provides a possible explanation for potassium and 4-AP evoked transmitter release to not be entirely dependent on external Ca^{2+}.

The selectivity of NMDA to specifically evoke Glu release suggests a distinct localization of EAA receptors on Glu neurons or a unique interaction of NMDA with the Glu transporter. The maximum effect of NMDA on Glu release was achieved only at a concentration of 3 mM which may be a significantly higher concentration than that required to normally activate NMDA receptors. NMDA was shown to inhibit [^{3}H]-Glu binding in rat brain postsynaptic densities with a K_i of 7.2 μM (Fagg and Matus, 1984) and stimulate GABA release and [^{3}H]-GABA release from striatal cultures with EC50's of 19 μM and 22 μM, respectively (Pin et al., 1988; and Jouanen et al., 1991). At a concentration of 3 mM, NMDA may be activating AMPA receptors or altering the uptake of Glu. However, I was unable to show that AMPA could release Glu or GABA from the tissue slices, and selective Glu receptor antagonism in the presence of NMDA receptor stimulation was not explored; this would have deviated from the central focus of the project.

Much controversy exists as to whether NMDA receptors are expressed presynaptically in the striatum. Greenamyre and Young (1989) use autoradiography and lesion studies to report that the majority of NMDA receptors in the striatum are located postsynaptically, and they provided no support that NMDA receptors are presynaptically located on Glu terminals. However, Ohta et al (1994) showed evidence suggesting that NMDA as well as AMPA receptors exist on monoaminergic terminals throughout the striatum, and Young and Bradford (1991) and Errington et al (1987) produced pharmacological evidence indicating that NMDA receptors are on Glu terminals to mediate an apparent "positive feedback" on Glu release. Two lines of evidence suggest that NMDA stimulation of Glu release was not receptor mediated: I showed NMDA stimulation of Glu release to be Ca^{2+}-independent and insensitive to the presence of Mg^{2+}. This does not necessarily preclude a receptor mediated action of NMDA. However, because NMDA only released Glu and not GABA, it is likely that NMDA was acting on the synaptic Glu transporter and releasing Glu through reversal of the transporter. NMDA receptor stimulation, in the absence of external Ca^{2+}, can evoke depolarization-induced reversal of the presynaptic transporter by increasing the internal Na^+ concentration (Weiss, 1988a; Pin and Bockaert, 1989: Weiss, 1990; and Bernath, 1992). Large amounts of NMDA may stimulate AMPA receptors enough to relieve the Mg^{2+} block on local NMDA receptors, thereby increasing Na^+ influx through the AMPA or NMDA receptor channel. Also, I found that 1 and 3 mM NMDA stimulation of Glu release increased approximately 6-fold in the absence of external Ca^{2+}, and that basal GABA release by these concentrations of NMDA was increased by over 12-fold (data not shown). This may be indicative of Na^+-dependent, depolarization-induced reversal of presynaptic transporters as discussed above, whereby large amounts of Na^+ entered the neuron through voltage-sensitive Ca^{2+} channels that were unoccupied by external Ca^{2+} (Bernath, 1992).

The insensitivity of GABA release from the slices by NMDA and the selective actions of 4-AP on GABA release requires some explanation. Although GABAergic medium-size

spiny neurons in the striatum are thought to possess NMDA receptors, Di Chiara et al (1994) proposed that NMDA-receptor influence of GABA activity is most likely mediated indirectly through Glu's primary action on NMDA receptors located on cholinergic neurons. GABA is the "fast inhibitory transmitter" in the striatum and receives fast excitatory input from Glu which utilizes AMPA receptors; NMDA receptors mediate a slower response and function in a modulatory role in the striatum, much like DA and ACh do (Di Chiara et al., 1994). It is likely that the tissue slices under my experimental conditions were unable to show "modulatory" activity of NMDA receptor stimulation: incubating the tissue for 5 minutes with such large amounts of NMDA was not optimal conditions for obtaining either NMDA evoked cholinergic activity or release of enough ACh by NMDA to influence GABA release. 4-AP, on the other hand, was a very effective stimulator of GABA release; basal release increased nearly 5-fold with 1.0 mM 4-AP. GABA release, arising from interneurons, recurrent collaterals and dendrites, may have a greater sensitivity to voltage-sensitive potassium channel blockade, while Glu release, which comes entirely from terminals of long efferent neurons, may express less of the 4-AP-sensitive potassium channels.

C. Significance of presynaptic reuptake on potassium-evoked release of glutamate and GABA from rat caudate-putamen tissue slices

Both Glu and GABA are removed from the synaptic extracellular space by specific reuptake transporters that re-accumulate the amino acids back into the presynaptic terminal or into surrounding glia (Schousboe et al., 1979; and Robinson et al., 1991). I studied the effects of specific Glu and GABA reuptake inhibitors on potassium stimulated release of the amino acids to determine if presynaptic reuptake played a significant role in the tissue slice release assay. The stimulated release of both Glu and GABA was shown to increase significantly in the presence of their selective reuptake blockers, and this effect was shown to be fairly selective. Nipecotic acid significantly increased the potassium- (50 mM)

stimulated release of GABA by over 2-fold at all concentrations tested and had little effect on Glu release. A slight, yet statistically significant increase in Glu release was observed at the highest concentration of L-trans-2,4-PDC. This did not necessarily suggest, though, that a *significant* level of released amino acids were being transported into their respective synapse in the absence of the transporters.

Further studies were necessary to determine if nipecotic acid or L-trans-2,4-PDC were mediating their effects through transporter inhibition and not through uptake and transmitter displacement. Both blockers caused an increase in basal release of the amino acids. Nipecotic acid increased basal GABA release in a concentration-dependent manner to over 12-fold at its highest concentration, and basal Glu release was increased by 2-fold with 30 μM L-trans-2,4-PDC. The effect of nipecotic acid on both stimulated and basal release of GABA was also shown not to be dependent on Ca^{2+}, indicating that the observed effects were not through depolarization-induced exocytotic release. It is important to note that the stimulatory effects of both uptake inhibitors on basal release was greater than their effects on 50 mM KCl stimulated release. This, again, suggests that their effects on release were not a result of uptake inhibition; it is unlikely that more transmitter was being taken up during basal release than during stimulated release.

Previous reports have shown that nipecotic acid, the prototypic GABA uptake inhibitor, possesses substrate affinity for the transporter and is capable of releasing GABA from neuronal stores (Johnston et al., 1976; and Waldmeier et al., 1992). Nipecotic acid itself is taken up into GABAergic neurons and released by potassium depolarization in a Ca^{2+}-dependent manner (Johnston et al., 1976), suggesting that it incorporates into synaptic vesicles and displaces GABA into the synaptic cytoplasm. L-trans-2,4-PDC, too, despite its high potency and preferential use as a Glu neuronal reuptake inhibitor, has been reported to possess substrate affinity for the Glu transporter and to release neuronal stores of Glu (Waldmeier et al., 1993; and Kanai et al., 1994).

These results, combined with reports discussed above, led me to consider that the significant increases in release of the amino acids observed in the presence of their respective uptake inhibitors was not a result of significant reuptake inhibition, but rather the release-evoking effects of the transporter blockers. These considerations, however, do not rule out the importance of transmitter reuptake *in vivo* or in the experimental design, but blockers with greater specificity were not commercially available, so further studies on uptake inhibition were not explored.

Amino acid neurotransmitter synaptic reuptake transporters are thought to operate less efficiently under elevated potassium depolarization because of significant alterations in the Na^+ concentration gradients and membrane potential and inhibition of Na^+ binding to the transporter (Bernath, 1992). Also, GABA release by the voltage-dependent potassium channel blocker, 4-AP, is accompanied by prolonged depolarization of the neuron (Thesleff, 1980), and this significant alteration in the membrane potential is likely to reduce the Na^+ concentration gradient necessary for GABA transport. Hence, potassium depolarization-evoked release of Glu and GABA, or GABA release evoked by 4-AP is most likely not altered in a significant manner by active presynaptic transporter reuptake.

D. P-type calcium channel involvement in the calcium-dependent release of glutamate and GABA from rat caudate-putamen tissue slices

The specific Ca^{2+} channels involved in the potassium- and 4-AP- evoked Ca^{2+}-dependent release of Glu and GABA from the CP tissue slices were investigated to determine the primary origins of the released amino acids. Three major classes of Ca^{2+} channels in the CNS are the L-, N- and P- types, and as discussed in the *Introduction*, they are thought to be expressed on specific areas of central neurons. Because L-type channels are uniquely located on proximal dendrites and the soma, and not the axon (unlike the N- and P- types which are located on the entire neuron including the axon), I had proposed that proximal dendritic release of GABA could be identified by its sensitivity to nifedipine,

the L-type channel blocker. Glu release, arising only from the axons of efferent neurons, should not be sensitive to nifedipine and only sensitive to either N-type blockade by CnTx GVIA or the P-type blockade of AgTx IVa.

I showed that only AgTx IVa was effective at significantly reducing the Ca^{2+}-dependent release of Glu and GABA by KCl depolarization and GABA release by 4-AP. Contrary to my predictions, nifedipine had no significant inhibitory effects on GABA release and CnTx GVIA failed to alter either amino acid release significantly. AgTx IVa (1 μM) reduced 50 mM KCl stimulated release of Glu and GABA to 33 ±5% and 18 ±0% control release, respectively; GABA release by 0.3 and 1.0 mM 4-AP was reduced to 51 ±6% and 40 ±5% control release, respectively. The inhibitory effects of 1 μM AgTx IVa reached statistical significance ($P<0.005$) in all cases, and when compared to initial Ca^{2+}-dependency studies ($CaCl_2$ removed from the media and 1 mM EGTA added), all Ca^{2+}-dependent release by potassium depolarization appears to be entirely contingent upon external Ca^{2+} influx through the P-type voltage-sensitive Ca^{2+} channel. For example, I showed that in the absence of external Ca^{2+} with EGTA present, 50 mM KCl stimulated release of Glu and GABA was reduced to 31 ±0.3% and 17 ±1.2% control release, respectively; this is very comparable to 33 ±5% and 18 ±0% control release for Glu and GABA, respectively, under 50 mM KCl stimulation with external Ca^{2+} and AgTx IVa present. The Ca^{2+}-dependent release of GABA by 4-AP depolarization also appeared to be entirely dependent on Ca^{2+} influx through the P-type Ca^{2+} channel. GABA release by 0.3 mM and 1.0 mM 4-AP was reduced to 43 ±2% and 44 ±9% control release, respectively, with external Ca^{2+} removed, compared to 51 ±6% and 40 ±5% control release, respectively, with external Ca^{2+} and AgTx IVa present.

The insensitivity of Ca^{2+}-dependent GABA release to nifedipine does not necessarily indicate that GABA release under these experimental conditions was not of a dendritic source. It does however show that L-type Ca^{2+} channels were most likely not involved in the Ca^{2+}-dependent release of the amino acids. Simmons et al (1995) showed dendritic

DYN release to be specifically sensitive L- and N- type Ca^{2+} channel blockade, while axonal release was only sensitive to N-type channel blockade. This was performed, however, using electrical stimulation of hippocampal tissue and under various other different experimental conditions. In the CP, dendritic release of GABA may occur and probably does occur *in vivo*, and the L-type Ca^{2+} channel may play a role in Ca^{2+}-dependent transmitter release; but under my experimental conditions, the Ca^{2+}-dependent release of GABA was solely dependent on Ca^{2+} influx through the P-type channel, and the L-type channel, according to Hell et al (1993), may have only performed its role in cellular functions such as gene expression and enzyme activity, not transmitter release. As discussed in the *Introduction*, previous reports showed that in rat striatal synaptosomes only N-type Ca^{2+} channels were involved in Ca^{2+} dependent release of [^{3}H]-GABA (Lecharny et al., 1995), and the P-type, and not the N-type of Ca^{2+} channel, was involved in Ca^{2+}-dependent release of [^{3}H]-Glu (Turner et al., 1993). Endogenous Glu release from striatal slices was also shown to be primarily dependent on Ca^{2+} influx through the P-type channel (Kimura et al., 1995).

E. Local transmitter influence on amino acid release from rat caudate-putamen tissue slices through actions of specific receptor ligands

50 mM KCl was shown to be an effective depolarizing stimulus of Glu and GABA release, however, elevated potassium is not a specific release stimulus: other striatal transmitters such as DA, ACh and opiate peptides were also likely released under potassium depolarization of the CP tissue slices. The analysis of these released transmitters was not in the scope of my project; however, I did intend to evaluated their influence on the measured release of Glu and GABA. Antagonists of muscarinic-ACh, DA, opiate peptides as well as Glu and GABA receptors were used to block effects, if any, of striatal transmitters on potassium stimulated release of the amino acids. A striatal transmitter was

thought to affect Glu and/or GABA release if that transmitter's receptor antagonist increased or decreased the measured amount of Glu and GABA in the tissue releasate.

Of the receptor antagonists assayed, atropine, saclofen, bicuculline, and SCH 23390 showed significant effects on release. Atropine specifically reduced Glu release by as much as 50% indicating a possible cholinergic influence on the stimulated release of Glu. The $GABA_B$ receptor antagonist, saclofen blocked release of both amino acids. This antagonist effect indicated, in this release assay, that the released GABA was not acting on $GABA_B$ autoreceptors to inhibit its release presynaptically; if it were, GABA release would have increased in the presence of the antagonist. Glu release was also reduced by bicuculline, the $GABA_A$ receptor antagonist, suggesting that the released GABA may have indirectly affected Glu release through some disinhibitory mechanism. For example, if 5-HT is inhibitory on Glu release in the striatum, and in turn GABA reduces the 5-HT input to the Glu neurons through both types of GABA receptors, then blocking these receptors would have relieved the GABA inhibition of 5-HT and thereby enabled 5-HT to reduce Glu release. A similar situation may be the reason for saclofen reducing GABA release, except that the disinhibition of the input into the GABA neurons is mediated only through the $GABA_A$ receptor. A dopaminergic influence on Glu release within this assay may be indicative of the results with SCH 23390, however the inhibitory effect of the D_1 DA antagonist was not concentration-dependent within the two concentrations tested. DA is most likely released with Glu, GABA and ACh following potassium depolarization, and it may have been acting on Glu neurons through the D_1 DA receptors to augment Glu release. By blocking these receptors, DA influence on Glu release was removed and Glu release decreased.

Because significant effects of the antagonists tested above were inhibitory toward release, it seemed likely that their corresponding receptor agonist would stimulate release. With exception of the GABA receptor antagonists, the direct effects of the antagonists could be interpreted so that bethanechol, a muscarinic-ACh receptor agonist, and 6-Br-

APB, the D_1 DA receptor agonist, would likely stimulate Glu release in the tissue slice assay. Both receptor agonists, however, failed to release Glu or GABA to levels significantly above basal release. Thus, under these experimental conditions, the inhibitory effects of the antagonists do not necessarily suggest that their corresponding receptor agonists elicit the opposite effect (i.e. stimulate Glu release). Of the opiate receptor agonists tested for their inhibitory role on potassium stimulated release, only the δ_1-agonist, DPDPE, yielded statistically significant effects, yet these effects were not concentration-dependent. It was especially surprising that neither μ-opiate receptor or D_1 DA receptor stimulation yielded any significant effects on GABA release, considering the localization of μ-opiate and D_1 DA receptors in the striatum.

F. GABA release from fetal rat primary striatal cultures

In view of the complex regulation of transmitter release in adult CP and the difficulty in observing inhibitory regulation of GABA release, the regulation of GABA release from primary cultures of embryonic rat striatal neurons was explored.

1. Release of GABA by depolarizing stimuli

Significant levels of GABA were released from the PSC by potassium, 4-AP and Glu. Release evoked by 50 mM KCl and 0.3 and 1.0 mM 4-AP was most likely through exocytosis because a majority of the release was Ca^{2+}-dependent; release stimulated by Glu, however, appeared less dependent on external Ca^{2+}. Release evoked by 10 μM Glu, the smallest concentration tested, was Ca^{2+}-dependent and reached a 3-fold increase in basal GABA release, similar to release stimulated by 30 mM KCl. However, release was independent of external Ca^{2+} when stimulated by larger amounts of Glu (25, 50 and 100 μM), and this release was slightly larger than release stimulated by 80 mM KCl, the most effective concentration of KCl for potassium depolarization. Of the three depolarizing agents tested, Glu was the most efficacious stimulus of GABA release, while potassium was clearly the most effective stimulator of Ca^{2+}-dependent release under these

experimental conditions. It is not surprising that Glu evoked Ca^{2+}-independent release of GABA from the PSC: in fetal mouse cultures, EAAs were shown to evoke Ca^{2+}-independent release of both [3H]-GABA (Weiss, 1988a) and endogenous GABA (Pin and Bockaert, 1989). Weiss (1988a) and Pin and Bockaert (1989) also showed that Na^+ was clearly essential for this release to occur, and that the Ca^{2+}-independent/Na^+-dependent release was due to reversal of the GABA transporter. Normally, GABA influx through the transporter is driven by inward electrogenic Na^+ and Cl^- gradients: 2 Na^+ and 1 Cl^- per molecule of GABA. When large amounts of Na^+ accumulate in the neuronal cytoplasm from Glu receptor activation, the transporter is reversed to pump Na^+ and Cl^- out, as well as GABA which travels with the electrogenic gradient (Pin and Bockaert, 1989). The two types of GABA release, Ca^{2+}-dependent and -independent, are shown below in figure 27.

Ca^{2+}-dependent release results in exocytosis from vesicular stores, while Ca^{2+}-independent release results from cytoplasmic pools of GABA released through reversal of the transporter. It is likely, although not fully shown, that Glu evoked Ca^{2+}-independent release of GABA in this culture preparation was through depolarization-evoked reversal of the GABA transporter.

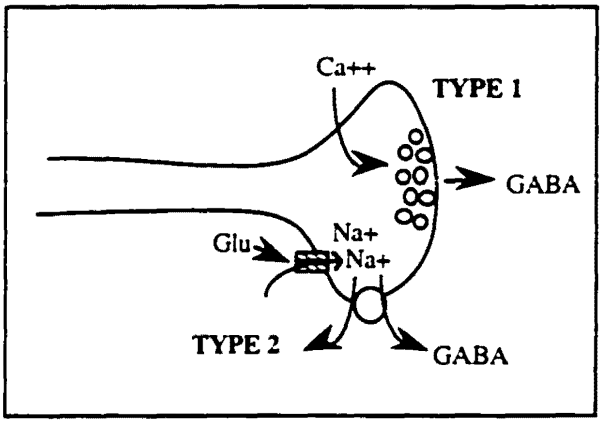


FIGURE 27 - Schematic representation of 2 major types of GABA release. Type 1 is calcium-dependent release from vesicles evoked by elevated KCl or 4-AP. Type 2 is independent of calcium, stimulated by Glu or other EAAs and mediated through reversal of the transporter. Adapted from Pin and Bockaert, 1989.

2. Dopamine receptor stimulation of GABA release

The D_1 DA receptor agonist, 6-Br-APB, stimulated the release of GABA to levels nearly 2-fold above basal release. While the lowest concentration tested, 3 nM, yielded no effect, both 30 and 60 nM were equally effective at evoking GABA release. A maximum effect was achieved by 30 nM, so further experiments were conducted to verify statistical significance ($P<0.01$) at this concentration. It seems likely that 30 nM 6-Br-APB is an ideal concentration to reach a stimulatory influence on GABA release, because its K_i for D_1 DA receptors is 1.37 nM (based on an IC50 of 4.3 nM; Neumeyer et al., 1992), and applying concentrations higher than 60 nM may have approached the lower levels at which D_2 DA receptors would have been activated by the agonist. The D_1 receptor antagonist, SCH 23390 (30 nM), effectively blocked the agonist's stimulatory actions on GABA release ($P<0.005$) suggesting that 6-Br-APB stimulation of GABA release was purely receptor mediated. This was likely a direct effect on GABA release because of the DA receptor localization on GABA neurons in the striatum (Gerfen, 1992), and the proposed *in vivo* role of DA on striatonigral/endopeduncular efferents of the striatum (Gerfen, 1992; and Di Chiara and Morelli, 1993).

6-Br-APB increased potassium (50 mM KCl) stimulated release of GABA by 22 ±8%, yet failed to reach statistical significance ($P<0.01$); GABA release by 4-AP was not affected by the agonist. A possible reason that D_1 receptor stimulation did not significantly increase depolarization-evoked GABA release, is that potassium-stimulation and D_1 DA receptor activation increase release by similar mechanisms. Stimulation of D_1 DA receptors activates adenylyl cyclase (Anderson and Jansen, 1990) which ultimately stimulates internal Ca^{2+} accumulation; however, under depolarization, internal Ca^{2+} accumulation has already been activated, and therefore D_1 receptor stimulation of adenylyl cyclase would have no potentiating effect. The effects of elevating intracellular Ca^{2+} on DA receptor function must also be considered. Neuronal adenylyl cyclase is activated by Ca^{2+} when Ca^{2+} is bound to calmodulin (Ross, 1990), and this may desensitize D_1 DA receptors.

The inhibitory D_2 DA receptor agonist, quinelorane (30 and 100 nM), had no significant effects on either 30 or 50 mM KCl stimulated release of GABA. 30 mM KCl was used after studies were first conducted with the higher concentration of KCl; I thought that inhibitory effects on release could be better analyzed under a smaller depolarizing stimulus. Stimulation of D_2 DA receptors results in inhibition of adenylyl cyclase, modulation of phosphoinositide metabolism, enhancement of potassium conductance and inhibition of Ca^{2+} entry through voltage-sensitive Ca^{2+} channels (Cooper et al., 1996). These activities combined may not elicit an effect great enough to achieve a measurable reduction in the potassium evoked release of GABA from the cultured neurons. Also, elevated potassium may impose such a large change in the ion conductance across the GABA neurons that no inhibitory effects were likely to be observed through D_2 receptor activation.

3. GABA and μ-opiate receptor activity on stimulated release of GABA

GABA release evoked by either 50 mM KCl or 0.3 mM 4-AP was not affected by the μ-opiate receptor agonist, DAMGO at 1 μM. Pharmacological evidence by Chneiweiss et al (1988) and Eriksson et al (1991) has shown that morphine and DAMGO inhibited adenylyl cyclase activity in fetal mouse striatal cultures which is suggestive of functional μ-opiate receptor activity in primary striatal cultures. The lack of DAMGO effects on stimulated GABA release in my experiments, may suggest that inhibitory actions of μ-opiate receptor stimulation are ineffective against powerful depolarizing agents such as potassium and 4-AP. As seen with quinelorane, the D_2 DA agonist, the depolarizing stimuli may alter the ion conductance of GABA neurons to a degree that cannot be affected by opiate receptor stimulation.

It is also possible that the released GABA might have been acting on striatal GABA receptors and thus be reducing the amount of GABA released by neuron depolarization. This might have masked any inhibitory effect of DAMGO. GABA receptor antagonists were added with the elevated potassium to determine if a significant amount GABA release

was being reduced by GABA receptor stimulation. Of the three antagonists tested only bicuculline, the $GABA_A$ receptor antagonist, was effective at significantly increasing the 50 mM KCl stimulated release of GABA. It appeared that GABA was released and acting on $GABA_A$ receptors to reduce its own release. A role for $GABA_B$ receptors is less likely, since neither saclofen nor 2-OH saclofen caused significant changes in GABA release. Kowalski et al (1995) have shown that in fetal rat primary striatal cultures, most of the spontaneous synaptic inputs of striatal neurons were mediated by GABA, and that these GABA-induced currents were through Cl^- channels and sensitive to bicuculline. This GABA mediated effect was insensitive to $GABA_B$ receptor antagonism and suggestive of $GABA_A$ receptor activity (Kowalski et al., 1995). It is possible that $GABA_B$ autoreceptor regulation of GABA release does not occur in the striatum, because as stated in the *Introduction*, $GABA_B$ receptors are thought not to be expressed on either the terminals of recurrent collaterals or on the GABA cell bodies in the striatum (Seabrook et al., 1991). Instead, in this striatal culture preparation, I showed autoreceptor regulation of GABA release to occur through $GABA_A$ receptors.

I was unable to show that DAMGO could completely block the bicuculline increase in potassium stimulated release of GABA. In six separate experiments, 100 μM bicuculline increased stimulated release to 143 ±18% control release ($P<0.05$) and 1 μM DAMGO, in five experiments, reduced this effect to 107 ±15%. Although an inhibitory trend appeared to occur, no statistical significance was established ($P>0.05$). It is possible that a weak inhibitory effect of μ-opiate receptor activation is masked by the variance in this assay system.

CONCLUSIONS

The work I have presented focused on the regulation of Glu and GABA release from rat CP tissue slices and GABA release from primary cultures of fetal rat striatal neurons. Several aspects of this project I believe are significant in the advancement of pharmacology and neuroscience research, all of which stem from the methods I employed to analyze tissue releasate levels of the amino acids and the results I obtained from the release experiments.

I have described an improved method of measuring tissue levels of Glu and GABA through LCED analysis of OPA-SO_3 derivatized amino acids. This method was initially used to measure amino acid release from tissue slices using an unique 96-well microtiter plate release assay. The methodology was recently published (Phillips and Cox, 1997) and has received interest from the Millipore Corporation for its use with their Multiscreen microtiter plate and filtration system. Glu and GABA release from CP tissue slices induced by potassium depolarization, and GABA release induced by 4-AP were shown to be Ca^{2+}-dependent, while the selective stimulation of Glu release by NMDA was not dependent on external Ca^{2+}. Release studies with selective Ca^{2+} channel antagonists showed that Ca^{2+} influx through the P-type Ca^{2+} channel was required of Ca^{2+}-dependent release, while Ca^{2+} influx through L- and N- type channels was not required of release. Surprisingly, ACh, DA and opiate receptor agonists failed to elicit effects on the release of Glu and GABA from the tissue slices. Primary striatal cultures (PSC) were employed as a more specific means of studying *in vitro* GABA release.

GABA release from PSC was evoked by potassium, 4-AP and Glu. Like NMDA stimulation of Glu release from the tissue slices, GABA release from the cultures evoked by Glu was independent of external Ca^{2+}. I have proposed that EAA-receptor stimulated release of the amino acids under these experimental conditions is mediated through transporter reversal and most likely requires internal Na^{+} accumulation with a resulting

reversal of the ionic gradient driving the amino acid transport. The release of GABA by D_1 DA receptor stimulation was significant in showing that DA exerts a stimulatory influence on striatal GABA neurons in culture, as has been proposed for *in vivo* DA regulation of striatonigral/endopeduncular neurons. Also, the bicuculline stimulated increase in potassium-evoked GABA release was important in confirming that autoreceptor regulation of GABA release in the striatum occurs through $GABA_A$ receptors and not the $GABA_B$ receptor, under these experimental conditions.

The results reported here may also lead to further investigations into the regulation of *in vitro* GABA release. In particular, because specific opiate receptors are co-localized with D_1 DA receptors on striatal GABAergic neurons, the functional role of their co-localization could be measured by testing if opiate receptor stimulation could decrease the GABA release evoked by D_1 DA receptor activation.

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IMAGE EVALUATION
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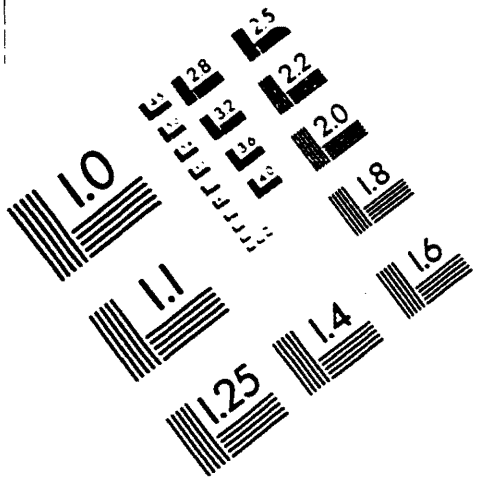

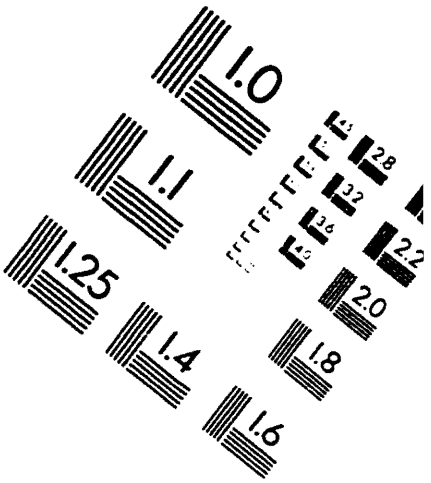

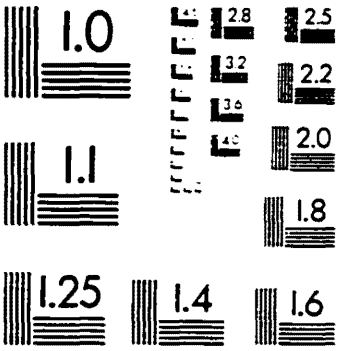

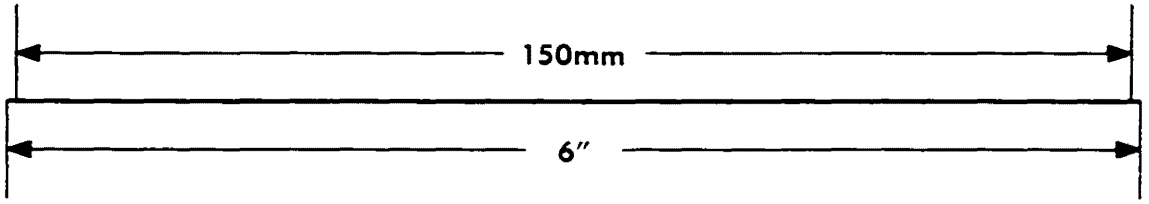

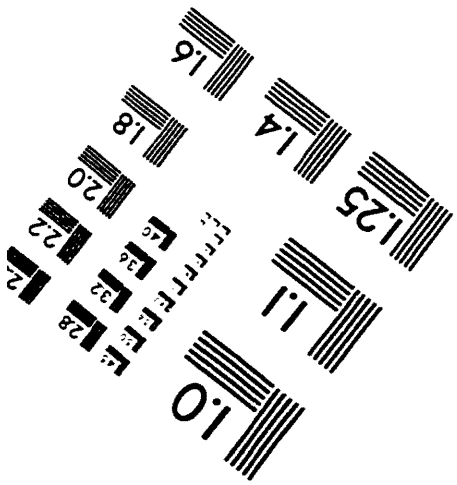

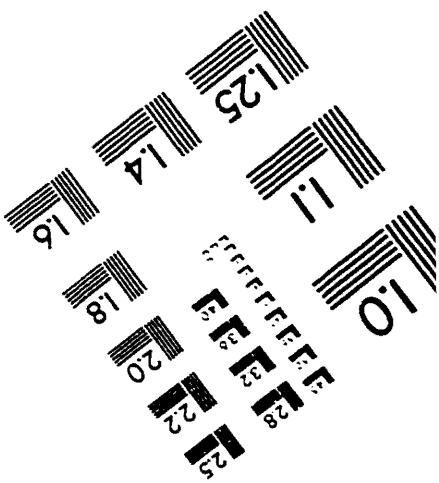

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